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TECHNIQUES AND RESOURCES

CLoNe is a new method to target single progenitors and study their progeny in mouse and chick

Fernando García-Moreno*, Navneet A. Vasistha, Jo Begbie and Zoltán Molnár*

ABSTRACT

Cell lineage analysis enables us to address pivotal questions relating to: the embryonic origin of cells and sibling cell relationships in the adult body; the contribution of progenitors activated after trauma or disease; and the comparison across species in evolutionary biology. To address such fundamental questions, several techniques for clonal labelling have been developed, each with its shortcomings. Here, we report a novel method, CLoNe that is designed to work in all vertebrate species and tissues. CLoNe uses a cocktail of labelling, targeting and transposition vectors that enables targeting of specific subpopulations of progenitor types with a combination of fluorophores resulting in multifluorescence that describes multiple clones per specimen. Furthermore, transposition into the genome ensures the longevity of cell labelling. We demonstrate the robustness of this technique in mouse and chick forebrain development, and show evidence that CLoNe will be broadly applicable to study clonal relationships in different tissues and species.

KEY WORDS: Clonal lineage, CRE recombinase, *piggyBac* transposase, Multi-fluorescence, Progenitor cells, Fate mapping, Electroporation, Cerebral cortex, Wulst, Dorsal ventricular ridge

INTRODUCTION

The goal of cell lineage analysis is to provide information on the number, distribution, phenotype and functional integration of all cells derived from the same single embryonic stem cell or progenitor population (Buckingham and Meilhac, 2011). Detailed clonal analysis has only been achieved in relatively simple organisms, such as *C. elegans* (Sulston, 1983), and fundamental questions remain to be answered in vertebrates despite considerable progress. The challenge is to selectively target a particular progenitor population within a defined sector of the developing embryo and follow the development and integration of the derived cells to adulthood. Methods are needed to study several clones synchronously *in vivo* as they integrate into functional networks. Moreover, to address the important questions related to comparative evolutionary developmental biology, the same methods should work in distinct species.

Most common clonal labelling methods are based on viral particles, such as retrovirus (Cepko et al., 1995; Heins et al., 2002; Li et al., 2012; Reid et al., 1995) or lentivirus (Sato et al., 2010),

carrying a reporter gene. However, these methods have drawbacks and could suffer from either underestimating or overestimating the size of the clones. To minimise such mistakes, very few clones must be studied in a given specimen, impeding the study of generation, integration and relationship of multiple clones.

New genetic models with cre-mediated recombination in specific cell populations enable the expression of reporter genes in a spatiotemporal manner (Danielian et al., 1998) facilitating the development of clonal lineage analysis methods from selected progenitors. Nkx2.1-expressing progenitors in the brain have been labelled in mice using retrovirus injections (Brown et al., 2011), yet reliance on a Cre mouse line restricted the experimental design to this species. The discovery and generation of various fluorescent proteins has greatly aided in the development of combinatorial multifluorescent labelling, allowing delineation of distinctly labelled fluorescent cells (Livet et al., 2007; Tsien, 2009). Original Brainbow methodology (Livet et al., 2007) was limited by default red fluorescence in non-Cre expressing cells. This initial shortcoming was recently amended (Cai et al., 2013) but still the use of Brainbow methods in clonal lineage analysis is extremely limited owing to the paucity of Cre-expressing chick and zebrafish lines. Electroporatable constructs cannot label entire lineages due to their incapacity to be integrated in the genome. However, its multifluorescent combination concept has been successfully applied to lineage analysis in *Drosophila* Brainbow (Hampel et al., 2011) and Star Track analysis of astrocyte clones (García-Marqués and López-Mascaraque, 2013). Unfortunately, Star Track relies upon a single promoter (*GFAP*) to select a specific progenitor population and for subsequent expression of the fluorophore. Hence, it is required that the promoter remains active throughout the entire period of investigation for fluorescent reporter expression. Although this continued expression is present in the particular case of astrocytes, this approach makes its use limited to other scenarios.

We have developed a new method, CLoNe (clonal labelling of neural progenies), to study clonal lineages, that tackles the disadvantages of other methods. We tested CLoNe on several tissues with a particular focus on forebrain neural progenitors and found that it provides a remarkably accurate and reliable fate-map of multiple clones originating from independent stem/progenitor cells within the same tissue volume of the same specimen. The method is particularly suited for *in vivo* and comparative studies on clonally related cell populations.

RESULTS

We developed the method of CLoNe to enable clonal lineage analysis of the progeny of specifically targeted progenitor cells. CLoNe consists of a mixture of three different kinds of plasmids (labelling vectors, targeting vector and transposase expressing cassette; Fig. 1) that, when transfected into cells, initiate a cascade of recombination events culminating in a unique colour code in each progenitor and its derived cells. The twelve labelling vectors each

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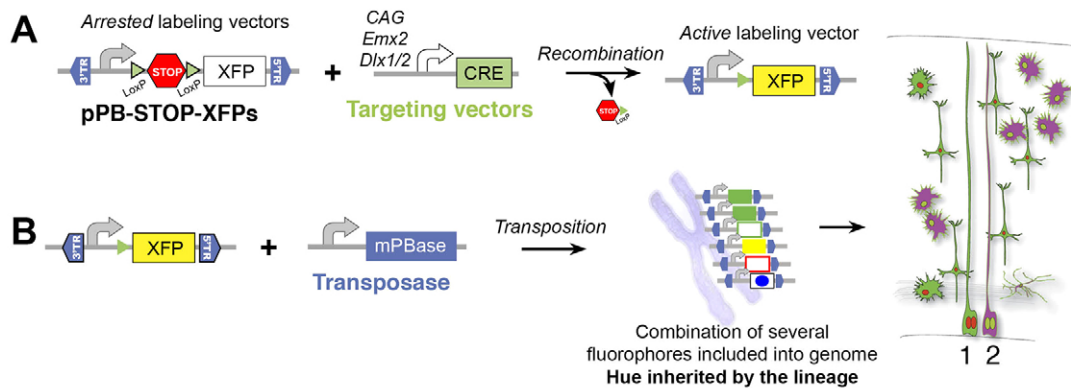


Fig. 1. Clonal labelling through CLONe. A mixture of three different types of vectors (labelling vectors, targeting vector and transposase expressing cassette) is co-transfected to the progenitors through electroporation. (A) Twelve plasmid labelling vectors, collectively named as pPB-STOP-XFPs, carry the expression of one fluoroprotein arrested by a loxP-flanked STOP signal. The targeting vector encodes the enzyme Cre recombinase under a promoter of our choice. Only progenitor cells expressing the selecting gene will express Cre, thus recombining the STOP and triggering the expression of the fluorophores by means of the strong constitutive promoter CAG. (B) Transposase enzyme recognises the 5' and 3' terminal repetitions at the labelling vectors, excises them and randomly integrates a small number of labelling cassettes into the genome of the progenitor. The random combination of fluoroproteins generates distinguishable hues at different subcellular compartments. These hues are permanent, stable and inheritable by the whole progenitor cell lineage.

contains one of four fluorophores together with one of three subcellular localisation signals downstream of a LoxP flanked STOP signal. The targeting vector encodes the enzyme Cre recombinase driven by regulatory control elements specific to the cell population of interest. Expression of Cre in this cell population results in removal of the STOP signal in the labelling vectors, allowing fluorescent protein expression. Finally the transposition vector encodes the enzyme *piggyBac* transposase (PB), which recognises specific terminal repeats flanking the fluorescent cassettes in the labelling vectors. PB transposase expression results in the random transposition of a small number of fluorescence cassettes into the genome (Woltjen et al., 2009). This genomic insertion is crucial as it creates stable labelling of transfected cells with a random, unique and heritable combination of fluorophore sequences. The thousands of possible combinations can be detected by confocal microscopy to reveal clonal relationships. We tested each of the different stages of the process, i.e. targeting of specific progenitors, transposition into the genome and generation of inheritable random combinatorial hues, to validate CLONe.

Targeting of specific progenitors

Labelling vector efficacy was tested in N2A cells. In the absence of Cre recombinase, no fluorescence was detected, demonstrating STOP signal function (supplementary material Fig. S1). However, on co-transfection with a constitutive Cre-expressing vector, fluorescence was seen (supplementary material Fig. S1). Furthermore, subcellular targeting of the tagged fluorophores to plasma membrane, cytoplasm or nucleus was also confirmed (supplementary material Fig. S1).

We tested eight candidate fluorophores and selected four based on a series of criteria (supplementary material Table S1). It was important that the fluorophores were stable and bright enough to be readily detected, with minimal overlap with other wavelengths. We also chose monomeric forms to prevent interactions between differentially tagged fluorophores.

For our method of clonal analysis to be advantageous, it is important to be able to target specific populations. We reasoned that the use of specific regulatory sequences would lead to Cre recombinase expression in a select progenitor population. However, as the regulatory sequences could also drive expression in earlier

iterations of the progenitors than those to be tested, we imposed a temporal control by using electroporation. During forebrain development, progenitors from a specific area express a unique code of regulatory genes (Guillemot et al., 2006; Kawaguchi et al., 2008) and in many cases, can be defined by expression of a single gene marker. We chose a number of these markers as candidates to label specific progenitors, and here show that a combination of their regulatory elements and *in utero* electroporation enables clonal analysis in subpopulations of forebrain progenitors.

Dlx1/Dlx2 are homologous genes highly expressed in the forebrain region that mainly generates the basal ganglia: the subpallium. The cis-regulatory elements driving *Dlx1/Dlx2* expression have been well described (Ghanem et al., 2007), and we selected the URE2 regulatory sequence to clone into the targeting vector. Electroporation of pURE2-CRE together with pPB-STOP-mCherry into mouse embryos at embryonic day (E) 12.5, early in forebrain development, resulted in the presence of red fluorescent cells at the subpallium only (Fig. 2A,B). By comparison, electroporation of pURE2-CRE:pPB-STOP-mCherry together with a ubiquitously expressing EGFP (pPB-CAG-EGFP) resulted in EGFP labelling of many progenitors at both pallial and subpallial levels (green cells in Fig. 2A), whereas only cells generated at the subpallium expressed mCherry. Thus, we could specifically label subpallial progenitors using pURE2-CRE.

A further aim in our design of CLONe was that it could be used across species. We therefore tested whether the same combination of vectors could be used to target a homologous progenitor population in the developing chick forebrain. Electroporation of the DNA constructs used above into chick embryos *in ovo* had identical results (Fig. 2C). Again, EGFP expression showed that though progenitors of both pallial and subpallial territories were transfected, yet only a small proportion of subpallial cells expressed mCherry. This demonstrates the ability of this technique to label specific progenitors across different species.

In addition to using CLONe to target different areas (Visel et al., 2013), we wanted to test its use to target different subpopulations of progenitors within the same area. In the developing forebrain, distinct progenitor subpopulations can be distinguished within the same region by expression of specific regulatory genes. For example, radial glial cells (RGCs) located at the neocortical

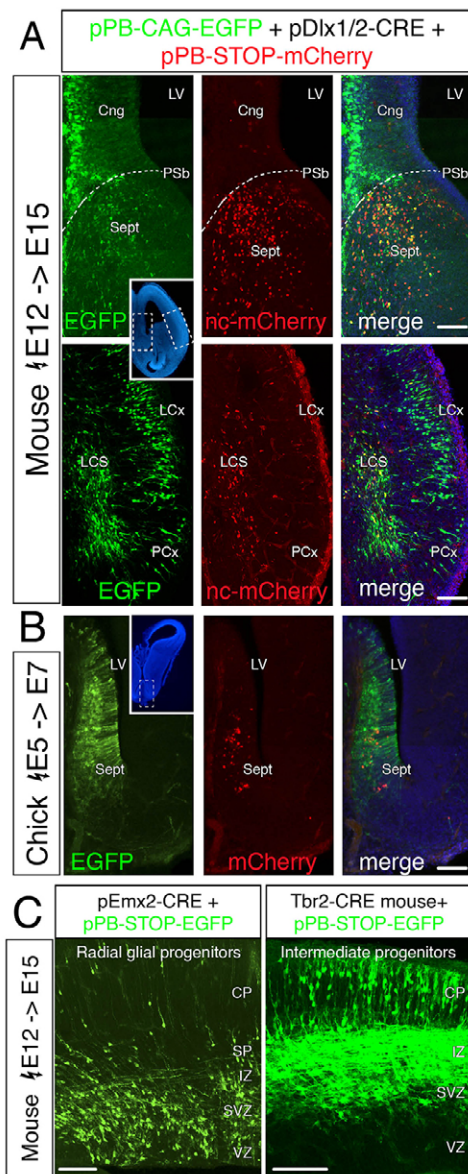


Fig. 2. Labelling of selected progenitors. A specific promoter that is active only in a selected region controls Cre expression and thus restricts the expression of fluoroproteins to targeted progenitors. (A) Examples of mouse forebrains electroporated at E12.5 and processed at E15.5 with a constitutive EGFP construct, pDlx1/2-CRE and pPB-STOP-mCherry. Examples are shown in the septum (sept, top row) and the lateral cortical stream (LCS, bottom row). Both pallial and subpallial progenitors were labelled with the electroporation, as demonstrated by green fluorescence. However, only subpallial cells at the septum and lateral cortical stream displayed red fluorescence. (B) Chick forebrains electroporated with the same cocktail as A. Same specificity of Dlx1/2 promoter targeted the expression of the red fluorochrome in subpallial cells only, whereas pallial co-transfected cells expressed only EGFP. Insets in A and B demonstrate the entire coronal section counterstained with DAPI at the level where images were taken from the regions depicted by white rectangles. (C) Selective Cre expression distinguishes radial glia and intermediate progenitors within the same sector of the brain. At the developing neocortex, radial glial progenitors express *Emx2*, its promoter selectively activates fluorophore expression in this progenitor population (left panel). Intermediate progenitors selectively express *Tbr2*, its promoter selectively activates fluorophore expression in intermediate progenitors, but not in radial glia. CP, cortical plate; IZ, intermediate zone; LV, lateral ventricle; PSb, pallial-subpallial boundary; SP, subpallium; VZ, ventricular zone; PCx, piriform cortex; Cng, cingulum, LCx, lateral cortex. Scale bars: 100 μ m.

ventricular zone express transcription factor *Emx2* (Cecchi, 2002) whereas intermediate progenitors (IPCs) at the subventricular zone (SVZ) are known to express *Tbr2* (Englund et al., 2005). Electroporation of a single labelling vector along with pEmx2-CRE (Suda et al., 2010) enabled the selective labelling of RGCs and their progeny (Fig. 2B). By contrast, electroporation of this labelling vector into mice directed fluorophore expression exclusively to IPCs but not to RGCs (Fig. 2C; N.A.V., F.G.-M., Siddharth Arora, Amanda F. P. Cheung, Sebastian J. Arnold, Elizabeth J. Robertson and Z.M., unpublished).

Efficient *piggyBac* transposition into the progenitor genome

The use of the *piggyBac* system to deliver transgenes into the genome has previously been described in mouse and chicken (Ding et al., 2005; Park and Han, 2012; Woltjen et al., 2009). Indeed, stable transposition of fluorophores into the genome has been employed to study the progeny of GFAP, GLAST and nestin-expressing lineages in the developing murine cortex (García-Marqués and López-Masaraque, 2013; Siddiqi et al., 2014). To determine the successful active transposition of labelling sequences using CLoNe, we addressed three criteria: first, whether fluorophore expression was maintained in the progenitors; second, whether the derived clones extended to different timed cohorts of the cortex; and, finally, whether late generated populations also acquired the fluorophore combination.

The maintenance of fluorophore expression in progenitors is evidence of successful genomic integration as in its absence the labelling vectors would remain episomal and be diluted with each cell division and subsequently lost. This lack of long-term fluorescence in progenitors can be seen in previous studies that use traditional non-transposable electroporation (see Hatanaka et al., 2004). In our experiments, progenitors transfected at early stages of embryonic development (E12 in mouse and E5 in chick) showed fluorescent staining several days (Fig. 3) and even weeks (Fig. 4F,G; Fig. 5) after transfection. In addition, the acquired unique combinatorial hue in the progenitor was preserved after mitosis, and daughter cells inherited the same fluorescent tag at either membrane, cytoplasmic and nuclear localisation (Fig. 3). This is significant because episomal vectors can be asymmetrically distributed between daughter cells during cell division, resulting in progenies with different hues. By contrast, genomic integration would result in faithful replication of the progenitor hue. We studied forebrain progenitor pools (VZ/SVZ) after CLoNe electroporations of early mouse and chick embryos. In both systems we found maintenance of the unique combinatorial patterns. In mice, analysis of ventricular (Fig. 3A) and abventricular progenitors (Fig. 3B) showed groups of two or three cells shortly after mitosis. In chick there were more numerous clusters of same-coloured cells in the germinal ventricular zone (Fig. 3C-E; supplementary material Fig. S2) with up to nine cells in one of the examples.

Our second test of genomic integration was whether CLoNe labelled all cortical layers. Neurogenesis in cortex follows an inside-out pattern. Consequently, neurons born earlier locate in deeper cortical layers than later-born neurons. Studies using traditional non-transposable neocortical electroporation affect progenitors only transiently and capture a snapshot of cortical neurogenesis, i.e. only label cells located in one or few layers in the cortex (Siddiqi et al., 2014). Our analysis of mouse neocortical sections following early CLoNe electroporations demonstrated a distribution of fluorescent cells across the entire depth of the neocortex ($n=11$ successfully electroporated pups at E12 analysed from P0 to P29, all of them showed a whole-layered cortical distribution of the labelled cells;

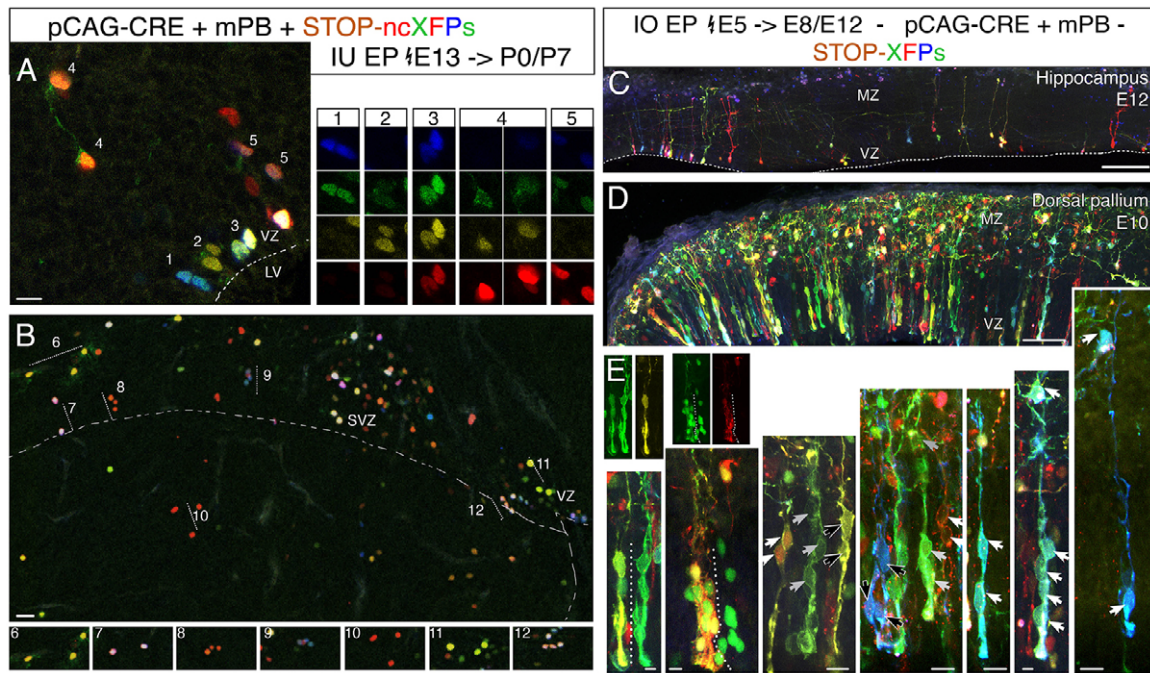


Fig. 3. Combinatorial hue remains stable after sequential mitoses in mouse and chick brains. (A-C) E13 mouse *in utero* neocortical electroporations of CLoNe vectors, with unrestricted selection (pCAG-CRE) and nuclear tagged fluorochromes, revealed at P0 (A,B) or P7 (C). (A) Cortical ventricular zone (VZ) at the dorsal forebrain at P0, 1 week after transfection. Five pairs of cells 6 days after transfection and just after mitosis shared the same combination of nuclear colours. Right panel shows images of isolated cells from left panel at the four different fluorescent channels. Eight pups were successfully electroporated and showed clonal labelling. The difference in intensity colour in pair of cells number 3 might be explained by a differential partition of the fluorescent proteins, which would be normalised once both cells display the fluorescent palette encoded by the genome. (B) Examples of conserved fluorescent nuclear hue after subventricular mitosis in the lateral telencephalon at P7, 2 weeks after electroporation ($n=8$, the eight animals showed clonal labelling). Groups of two or three cells, numbered 6 to 12, showed preservation of the fluorescent combination. (C-E) E5 chick electroporations of untargeted CLoNe vectors. (C) Progenitors labelled at the hippocampus displaying the high variability of fluorescent combinations. (D) Progenitor cells and postmitotic-derived cells at the dorsal pallium. (E) High magnifications of different progenitors from D and subsequent sections of the forebrain. Clusters of cells sharing the same shade of colour can be found at the VZ and in some cases at the mantle zone (MZ). Different arrows indicate clonally related cells derived from the same original stem cell; dashed lines separate clones. A detailed analysis of the fluorophore expression pattern and intensity hues can be found in supplementary material Fig. S2. Scale bars: 100 μm in C,D; 25 μm in B; 10 μm in A,E.

Fig. 4A). This lack of clustering indicates stable transfection of common progenitors for these cortical neurons with the fluorophores passed on to all daughter cells without dilution and attenuation of the label.

And third, we addressed whether early CLoNe electroporations continued to label late-generated populations during corticogenesis. In the developing cortex, glial cell populations are generated after neurogenesis, in early- to mid-postnatal development (Sauvageot and Stiles, 2002). Traditional non-transposable electroporation does not label these late populations as the episomal vector is lost (Siddiqi et al., 2014). By contrast, CLoNe electroporation in mouse was able to label astrocytes (in every pEmx2-CRE or pCAG-CRE driven CLoNe labelling after P5, $n=20$) and oligodendrocytes (in all pEmx2-CRE or pCAG-CRE driven CLoNe labelling after P10, $n=9$) during postnatal forebrain development, weeks after the transfection of the labelling vectors (Fig. 4B-G). The labelling appears stable as cells remain fluorescent at least 10 weeks after transfection (data not shown).

To rule out contribution from episomal copies of labelling vectors, we compared fluorescence expression of two labelling vectors: mCherry with intact PB repeats and EYFP lacking the 3' PB repeat and therefore incapable of genomic transposition (representing genomic and episomal contributions, respectively). As soon as 2 days after electroporation and continued at 7 and 14 days after transfection (supplementary material Fig. S3), cells expressing mCherry were found across different transfected areas (neocortex and striatum, $n=8$; supplementary material Fig. S3), whereas

expression of EYFP could not be detected. As previously described (García-Marqués and López-Mascaraque, 2013), this shows that transposition is necessary for the expression of fluorescence. Episomal vectors neither contribute nor alter mature genomic hue, which supported the presence of a single combination in each clone.

It is always possible that the integration site is deleterious or alters cell behaviour, as it could theoretically happen in other clonal methods such as viral infection. We considered this possibility real, but of very low probability. We have no information about the frequency of aberrant behaviours. However, we did not notice any evident alteration as cells always divided, migrated and differentiated in the previously described and expected fashion, and the somatodendritic morphology, projections, laminar position of the pyramidal neurons was in accordance with our expectation. In addition, the distribution of the analysed clones was similar to that found in previous literature.

These data demonstrate that our *piggyBac*-based method produced an effective and necessary transposition of the labelling sequences into actively transcribed regions of the genome for stable and permanent labelling of lineages.

Fluorescent hue is stable in postmitotic cells and reveals clonal relationship

Having successfully established the targeting and transposition steps of the labelling, we employed CLoNe in mouse and chick forebrain development to reveal clonal relationships. We defined a clone as a group of cells sharing the same combination of four different

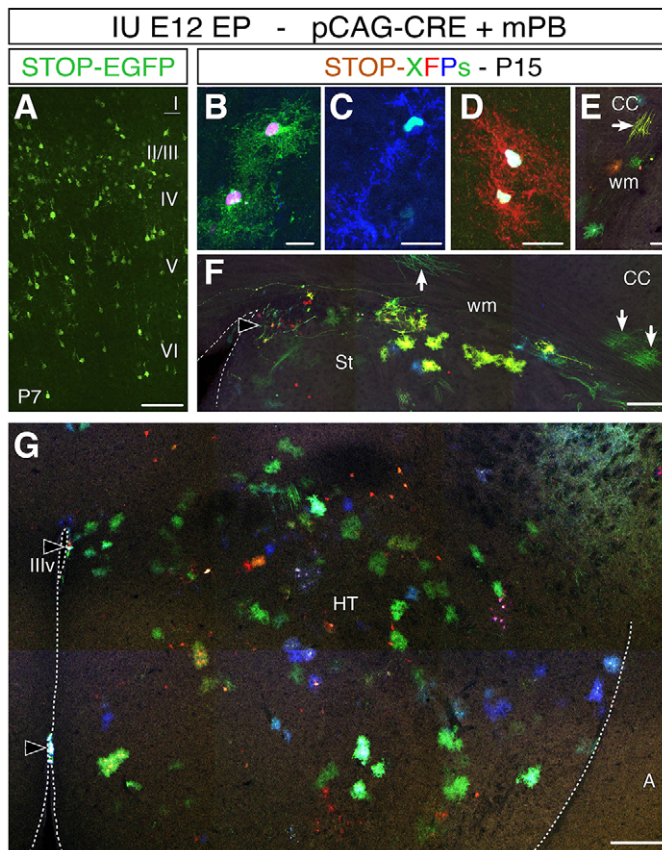


Fig. 4. Efficient *piggyBac* transposition into the genome allows the investigation of late derivatives of clones during postnatal stages. Untargeted CLoNe labelling demonstrates actual transposition of labelling cassettes. Mice were electroporated at E12 and brains were examined during postnatal development (A, P7; B-G, P15). (A) Neocortex of single arrested colour (STOP-EGFP) evidenced neurons located at layers II to VI of the neocortex at P7. (B-F) CLoNe labels clonally related astrocytes (B-D) and oligodendrocytes (white arrows; E,F) during late postnatal development (P15). Progenitor stem cells are still detectable in the VZ/SVZ (or subependymal zone) at the pallial-subpallial boundary (grey arrowhead) at P15. (G) CLoNe at the hypothalamus (HT) revealed many late-born astrocytes and multifluorescence at the progenitors (arrowheads) aligning the third ventricle (IIIv) at P15. A, amygdala; CC, cerebral cortex; wm, white matter; St, striatum. Scale bars: 200 μ m in G; 100 μ m in A,F; 50 μ m in E; 20 μ m in B-D.

fluorophores in their three possible tagged-forms. We reasoned that a broad spectrum of colours would be generated as most transfected progenitors acquire one or more copies of each labelling vector, while PB transposition creates a unique combinatorial hue (more than 1296 combinations in each of the compartments, as discussed below) by random genomic insertion of, on average, nine labelling sequences (Woltjen et al., 2009). This large number of potential combinations allowed us to remove spatial clustering as a requirement of clonal assessment. We analysed a single section of an E10 chick electroporated at E5 with pCAG-CRE driven CLoNe vectors using IMARIS (Bitplane) software (supplementary material Fig. S4). We spotted more than 1000 cells each expressing at least one fluoroprotein, with the IMARIS counted for EGFP being 1072 cells. The mean intensity of these EGFP-expressing cells was distributed equally across the four channels (supplementary material Fig. S4), suggesting an equal expression of the different fluorophores. Next, we determined the power of CLoNe to generate

and discriminate unique colours, considering that highly frequent combinations are not useful for clonal analysis. We thresholded the intensity into six levels and classified the mean intensity of each cell in each of the channels. Accordingly, each cell was assigned a colour code comprising four digits (the four colours, BGYR; from 0 to 5; it implies a number of 6^4 -1296 potential combinations). We recommend looking for and analysing clones displaying highly complex hues only, showing expression of more than one but not all the fluorophores in each of the compartments, rather than study single colour-labelled cells in either the nucleus or the membrane. In our experience, complex patterns tend to occur with the lowest frequency. When considering two independent hues in a given cell (nucleus and membrane/cytoplasm) and complex patterns only, the theoretical probability of two progenitors to acquire the same hue independently decreases to $1/6^4 \times 6^4 = 0.00005\%$. However, taking into account technical limitations (the brightness of two of the fluorophores is not entirely independent, confocal imaging limitations) in the practice and being conservative we can reliably discriminate at least 100 different hues per cellular compartment (analysis of Brainbow 1.0 mouse line L showed that from 89 to 166 colour hues can be identified with three different fluorophores) (Livet et al., 2007), so our estimate increases the likelihood of two progenitors to randomly acquire the same hue independently to 0.01%.

Our analysis detected up to 264 different colour codes (average of 4 cells/combination) in total cellular brightness measurement. Importantly 86 colour codes were represented in a single cell, and 96% of the codes (253 of 264) were represented in 15 or less cells; 795 out of 1072 cells (74%) belonged to low-represented codes (supplementary material Fig. S4). This low occurrence of the hues, and the different hue in either nucleus and membrane of the cell, made analysis of the random expression of these 12 fluorophores sufficient to discriminate clonal relationships. To further facilitate clonal analysis, we reduced the number of labelled progenitors by decreasing the targeting vector concentration (supplementary material Fig. S5) and also increased the variety of fluorophores. However, none of the additional fluorophores tested (mKeima, mCerulean, mKO and mTFP1; supplementary material Table S1) fulfilled the exhaustive requirements of brightness and independence of signal.

Early mouse and chick embryos electroporated with different combinations, proportions and concentrations of CLoNe vectors were analysed at several stages of embryonic and postnatal development with particular attention to clones (Figs 5 and 6). We first studied short-term labelling at the derivatives of the wing limb bud (supplementary material Fig. S6). CLoNe labelling was able to distinguish clonal relationships at the fibroblast and muscle cell lineages. In embryonic mouse forebrain, the *Emx2* promoter labelled clones in the neocortex and olfactory cortex (Fig. 5; supplementary material Figs S7 and S8). These clones were observed at P15, 3 weeks after the electroporation and abounded in the astrocytic lineage. We consider the main reason for the preferential astrocytic labelling is the differences in cellular shape and membrane morphology rather than preferential labelling pattern. Whereas membrane fluorophores tend to aggregate in the axonal component of neurons (Livet et al., 2007) and the morphology of the neuron is not well defined, the flat dispersed membranes of astrocytes and oligodendrocytes generate a more prominent and typical labelling that is easily identifiable. Clonal clusters of astrocytes were easily identified in all layers of the cerebral cortex (Fig. 5A) and in some cases dispersed across the entire depth of the cortical thickness (Fig. 5B, clones 5 and 6) but multifluorescence was also detected in

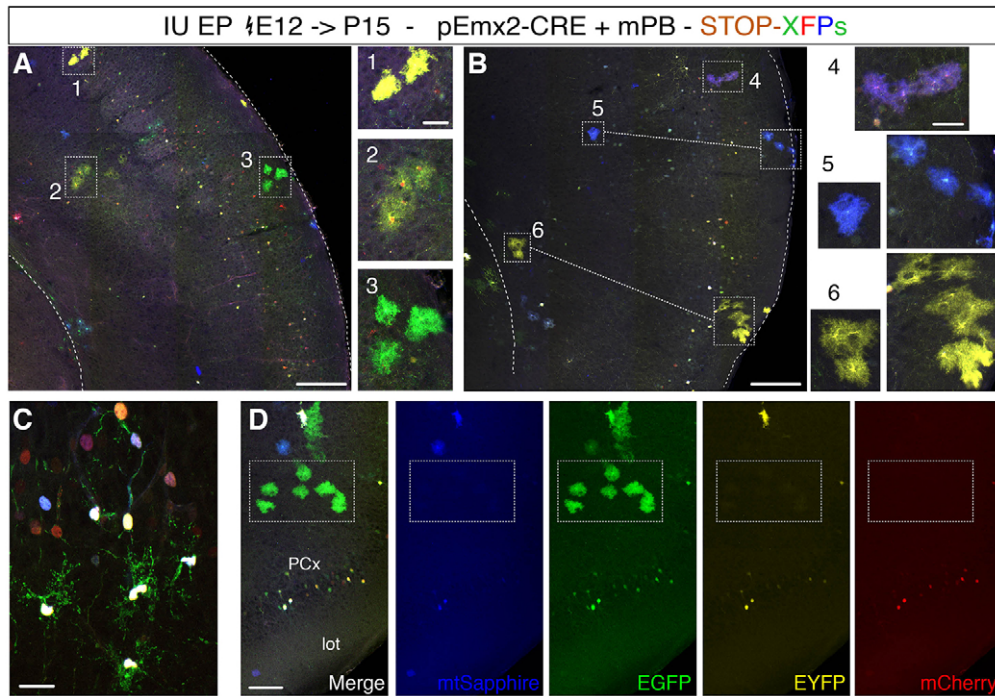


Fig. 5. CLoNe detects murine glial clones. Mouse E12 embryos were electroporated at the neocortical epithelium with CLoNe vectors targeted by *Emx2* promoter. Tissue was processed at P15. Six of the animals electroporated at E12 that survived up to P15 were actually labelled and five showed clonal labelling. (A,B) Lateral neocortex displays small groups of astrocytes stained with the same combinatorial hue. Clones 1-4 present clusters arranged in small cortical areas, whereas clones 5 and 6 show groups of cells separated by the cortical thickness. In clone 2, the membrane (green and yellow) and the nuclear labelling (red) are uniform among the four clonally related cells (see supplementary material Fig. S7). Dashed lines mark (right) the pial surface and (left) the white matter/cortex boundary. Dashed lines in B link the deeper and more superficial members of clones 5 and 6. Clones 1-6 are magnified in the insets at the right. (C) High magnification of a clone of four astrocytes labelled with mbEGFP and the four ncXFPs. (D) Merged and four independent channel images at the olfactory cortex showing a clone of six astrocytes only labelled with EGFP but not with the other fluoroproteins. Scale bars: 250 μ m in A,B; 100 μ m in D; 50 μ m (clone magnifications) and 25 μ m in C.

neurons (supplementary material Fig. S8). CLoNe labelling enables the quantification of several key features of these clones, such as clone size, cell types, cell morphology and location within the tissue, and allows us to postulate possible forms of cell migration or model possible involvement of different progenitors.

We found that the concentration of the targeting vector was crucial in determining the number of progenitor cells labelled with CLoNe. We assessed the optimal concentration for each targeting vector for clonal analysis and observed that a low concentration (\sim 10 ng/ μ l) of pCAG-CRE provides the best results (supplementary material Fig. S5). We performed our clonal analysis on chick forebrain development with these parameters and found multiple clones within the same tissue volume, allowing the study of the clonal inter-relationships (Fig. 6). Clones in the hyperpallium of E11 chick brain, 6 days after transfection, were selected according to the complexity of the combinatorial hue. The criteria for selection was that the cells must present the same independent nuclear and/or cytoplasmic labelling and must show a comparable brightness in each channel (supplementary material Fig. S9), avoiding mono-labelled (one single colour) or pan-labelled (all colour in all compartments) cells. After studying a total thickness of 500 μ m (Fig. 6A-G), we described five clones (Fig. 6H-L; supplementary material Fig. S9), with an average of seven cells per clone (from three cells in clone 3 to ten cells in clone 4). Importantly, membrane labelling allowed the analysis of detailed morphological and anatomical features of progeny. Hyperpallial clones were distributed radially across the entire thickness of the hyperpallium (Fig. 6M), with a tendency to locate in superficial levels of the hyperpallium in clone 5. Most of the cells

displayed a bipolar morphology without any preferred orientation. Remarkably clone 2 cells presented elongated neurites that extended towards other members of the same clone (Fig. 6M). As an important advantage, CLoNe allows the study of several targeted clones in the same region of tissue, enabling the investigation of interclonal relationships (supplementary material Fig. S10).

DISCUSSION

CLoNe provides a versatile tool to target specific progenitors and label all clonal cells in a unique and permanent fashion. The method builds on previous methods such as Brainbow, but it enables the labelling of several clones within the same volume of tissue using multicolor fluorescence supported by *piggyBac* transposition, which labels the entire clone for weeks and most likely for the entire lifespan of the animal. Moreover, CLoNe is based on the use of cell-type specific Cre. It allows comparative analysis of progenitor cells across species by using evolutionarily significant genetic elements to activate Cre expression. Furthermore, the use of plasmid vectors opens the possibility of genetically manipulating progenitors to study the impact of single genes on the clonal development of targeted populations. We propose that CLoNe is suitable for various tissues, as evidenced in muscular and epithelial tissue, and systems across several vertebrate species.

Selectivity of CLoNe relies on the selectivity of Cre expression

The most remarkable feature of CLoNe is the ability to discriminate progenitors and label their entire lineage. Transient expression of the

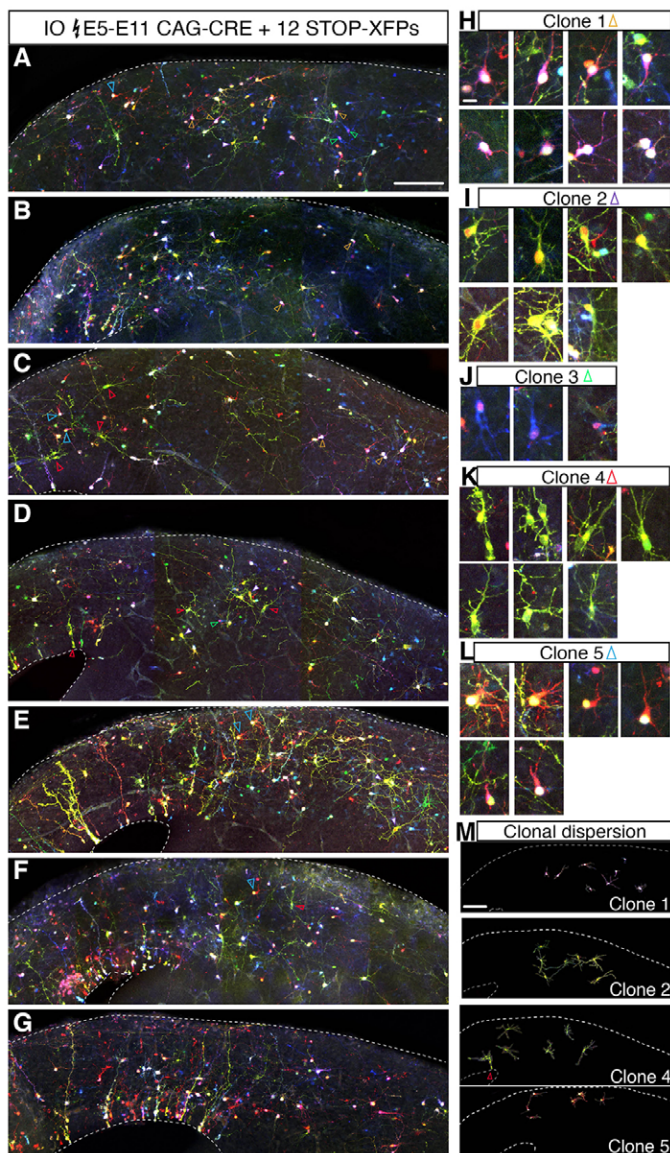


Fig. 6. Detection of chick neuronal clones. Chick E5 embryos were electroporated at the dorsal pallial epithelium with untargeted CLoNe vectors. Tissue was processed at E11. (A-G) Sequential sections at the forebrain hyperpallium. Many progenitors express fluoroproteins and their derived neurons share a common colour palette (colour arrowheads). (H-L) Higher-power images are shown of cells from the different clones. Members of each clone were pointed with coloured unfilled arrowhead of different shades of colour. For separate channels of all the cells of the five clones see supplementary material Fig. S9. (M) For the representation of the distribution of the elements of clones, individual cells belonging to a selected clone were isolated from the original image and presented selectively. Scale bars: 100 μ m in A-G,M; 10 μ m in H-L.

Cre gene ensures a controlled and reliable recombination of the labelling sequences. There are several features that are desirable in the promoter driving expression of *Cre* recombinase. *Cre* should be active for only one round of recombination, thus avoiding *Cre* recombinase toxicity and rare events of re-recombination that could alter the clonal hue in a given cell. Ideally, *Cre*-expression should be active only at a specific stage in a defined sector or cell population of the progenitor pool. In our example, *Emx2* is active during early neurogenesis in ventricular zone progenitors of the

developing cortex and switched off during mid-neurogenesis (Cecchi, 2002). Additionally, we grant a quickly transient *Cre* expression by transfecting a minimum concentration of the vector encoding the recombinase enzyme. An alternative solution when the promoter remains active is transient activation using the *CreERT2* system (Feil et al., 1997).

The targeting gene should be able to discriminate between different progenitors in a given area. In our study, *Tbr2* is expressed only in the intermediate progenitors of the SVZ (Englund et al., 2005) that arise from divisions of *Emx2*⁺ progenitors (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). As the vectors are passed on via the VZ, it is important that the *Tbr2* promoter be inactive in the VZ to avoid any labelling of the *Emx2*⁺ progenitor population.

Advantages of CLoNe over viral approaches for clonal labelling

Retroviral (Cepko et al., 1995; Heins et al., 2002; Li et al., 2012; Reid et al., 1995) and lentiviral particles (Sato et al., 2010) have been widely employed to label progenitor cells. However, as retroviruses integrate DNA in only one of the daughter cells after mitosis (Roe et al., 1993), they label only part of the clone, which could lead to underestimation of clone size. Lentiviral particles can also infect postmitotic cells (Bartholomae et al., 2011), thereby leading to overestimation of clone size. In both cases, a highly diluted titre of particles is needed to achieve restricted labelling of progenitors. The clones thus labelled must also be sufficiently separated in the tissue to allow reliable identification. Thus, analysis of relationship among closely located clones is impossible or requires tedious retroviral vector libraries carrying distinguishable DNA tags (Golden et al., 1995; Reid et al., 1995; Szele and Cepko, 1998). Importantly, the restrictive size of the reporter DNA (~5 kb) in viral particles narrows the potential for further genetic manipulations.

CLoNe tackles all these disadvantages. First, integrated DNA is inherited by both daughter cells, which abrogates underestimation. In the particular case of the developing brain, injection of plasmids into ventricles and oriented electroporation towards the brain surface enables transfection only in the ventricular zone where mitotic progenitors are present. Third, multifluorescence-based CLoNe also enables distinction between multiple clones per specimen, including long cellular migrations and other disperse clonal arrangements. Finally, this method can also be used in combination with gain- or loss-of-function experiments to study the effects on parameters of clones. As in every clonal analysis though, we recognise an impact of lumping and splitting errors. Whereas they might be associated with unexpected episomal fluorescence interference, uneven cellular distribution of fluorophores or least likely generation of identical hues in independent progenitors, we have not studied these in depth. However clones defined by our combinatorial labelling method most likely represent true biological clones.

piggyBac transposition allows clonal analysis

The CLoNe method is largely aided by use of *piggyBac* transposition (Ding et al., 2005; Wolftjen et al., 2009). It allows perpetuation of the label along with generation of random combinations. As transposition does not integrate all available copies into the genome, episomal copies can alter the hue and hence must be prevented. To achieve this, CLoNe employs a low concentration of labelling vectors that are further scattered in the progenitor pool by the need of *Cre* recombination. Thus, the number of labelling cassette copies available for transposition is greatly

reduced when compared with non-transposable electroporation. True to this, episomal constructs were not found in our long-term experiments, making it ideal for long-term fate mapping (García-Marqués and López-Mascaraque, 2013).

Owing to the very low probability of similar recombination pattern and thus similar colour spectrum, CLoNe is particularly valuable for the study of multiple clones within the same tissue volume. CLoNe can further benefit from various methods to make the tissue permeable; therefore, the reconstruction can be obtained from a larger volume of continuous tissue, rather than from reconstructed serial sections. These methods could help tackle other challenges related to the application of multicolor fluorescence to the study of lineages, such as potential intracellular fluorescence inconsistency or variable fluorescent brightness throughout increasing tissue depths. Combining CLoNe with OPT (MRC IGMM, University of Edinburgh), Scale (Hama et al., 2011), Clarity (Chung et al., 2013), SeeDB (Ke et al., 2013) or ClearT (Kuwajima et al., 2013) could be useful in these respects. Moreover, CLoNe could be combined with birthdating, which could help with the calculation and computation of cladograms of tagged cells.

CLoNe is well suited for various tissues and organisms to conduct comparative studies.

Originally CLoNe was developed to enable the study the progeny of selected progenitor populations in the cerebral cortex and the ganglionic eminences of mouse and chick and we have proven its suitability to other cell types such as fibroblasts and muscle syncytia. There was an emphasis to develop a method that could be used in sauropsids and mammals. Therefore, CLoNe is particularly suited for comparative studies when clones derived from specific progenitor populations are compared.

Development of this method also provides unique opportunities for embryology by being able to label the entire progeny of a progenitor population. To extract the information of such progenies and their relationships will require combination with new methods that allow imaging of larger tissue volumes as mentioned above.

We anticipate that several developmental communities will embrace this method because CLoNe is designed to work in all vertebrate species and tissues using the same constructs. As proposed CLoNe labelling can boost studies in broad biological sciences covering stem cell biology, cancer research and all areas of developmental biology.

MATERIALS AND METHODS

Plasmid vector generation

Plasmids were generated using standard cloning methods. Three different types of vectors are needed for the stable, selective and unique labelling of specific single progenitors and their progenies.

We started with pCAG-CRE (Matsuda and Cepko, 2007) (Addgene plasmid 13775; kindly provided by Dr Cepko, Harvard Medical School, Boston, USA) into which we replaced the CAG promoter with the mouse *Emx2* enhancer (Suda et al., 2010) (kindly provided by Dr Aizawa, RIKEN Kobe, Japan) or mouse *Dlx1/2* enhancer [URE2 sequence (Ghanem et al., 2007)] for subpallial progenitors. These sequences are 1-6 kb long and show a high degree of homology with the chicken genome.

Second, to generate the labelling vectors, a synthetic stop SV40 signal flanked by directly oriented LoxP sites was PCR amplified from the pBS302 plasmid (Sauer, 1993) (kindly provided by Dr Sauer, Oklahoma Medical Research Foundation, USA; Addgene plasmid 11925) and subcloned into a pPB-UbC-EGFP vector (Yusa et al., 2009) (kindly provided by Wellcome Trust Sanger Institute) between UbC promoter and EGFP sequence. The plasmid generated was pPB-UbC-loxP-STOP-loxP-EGFP and after replacement of the main promoter by CAG was referred to as pPB-STOP-

EGFP. The fluorescent protein in this first arrested vector was replaced with the different fluorophores detailed in supplementary material Table S1 (Ramakrishnan et al., 2000; Zapata-Hommer and Griesbeck, 2003; Livet et al., 2007; Kogure et al., 2008) to generate other labelling vectors. Either human H2B histone (GenBank ID X00088.1) or a palmitoylation sequence were cloned in frame at the 5' extreme of the fluorophore to tether XFPs to the nucleus or membrane, respectively. The resulting vectors were referred to as pPB-STOP-ncXFPs or pPB-STOP-mbXFPs. All plasmids were sequenced for confirmation.

Finally, the third vector required, mPB (Yusa et al., 2009) (kindly provided by Wellcome Trust Sanger Institute), expresses transposase activity that recognises terminal repetitions in pPB-STOP-XFPs, excises them and includes the labelling sequences into the genome. A truncated non-transposable labelling cassette (referred to as pCAG-STOP-EYFP) was generated by removing the 3' terminal repetition from pPB-CAG-STOP-EYFP.

All plasmids needed for CLoNe labelling are available through Addgene (<http://www.addgene.org/>).

Plasmid validation and N2A culture

All newly generated plasmid vectors were validated *in vitro* prior to their use *in vivo*.

Briefly, N2A cells were cultured in 25 or 75 cm² flasks at 37°C, 5% CO₂ in DMEM + Glutamax (Gibco) with 10% foetal bovine serum (Gibco) and penicillin/streptomycin (100 U and 100 µg, respectively; Gibco). Cells were passaged every two days by trypsinising with Trypsin-EDTA (0.25% trypsin) and centrifugation of the cells. N2A cells were transfected using FuGENE (Promega) in media without antibiotics. Fluorescence could be seen in N2A cells 24 hours after transfection.

As a final step, the efficacy of the vectors to label specific single progenitors and their whole progeny was tested *in vivo* by *in utero* electroporation.

CLoNe vector mixtures

DNA plasmids were dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and mixed with Fast Green (Sigma). Targeting vectors were used first at high concentration (0.5 to 1 µg/µl) and sequentially decreased to lessen the number of progenitors labelled (100, 10, 5 and 1 ng/µl of pCAG-CRE). Transposase vector (mPB) was consistently used at 300 ng/µl. Labelling vectors (pPB-STOP-XFPs) concentration varied according to the quality and brightness of the fluorescent signal, from 200 to 500 ng/µl each of the 12 vectors (around 3.5-4 µg/µl in total). Remarkably, best CLoNe performance was achieved when pCAG-CRE was transfected at 10 ng/µl, a concentration 30 times less than mPB. Whereas two vectors co-transfected at the same concentration can virtually reach a global co-transfection (Rana et al., 2004), the great difference between Cre-expressing and mPB vectors guarantees every cell transfected with Cre also expresses transposase. In our analysis, progenitors getting only Cre but not mPB do not display any fluorescence. Both factors together (large mPB:Cre vector ratio and lack of episomal cassettes-derived fluorescence) evidence that CLoNe reliably labels clones through *piggyBac* mediated transposition.

Animals

All animal experiments were approved by a local ethical review committee and conducted in accordance with personal and project licenses under the UK Animals (Scientific Procedures) Act (1986). Adult C57/BL6 mice were obtained from a local breeding colony at the University of Oxford [based on the Harlan (UK) strain]. They were maintained on a 12/12-hour light/dark cycle (7 am, lights on) and provided with *ad libitum* access to food and water. The day when vaginal plug was detected was referred to as E0.5.

Fertilised hens' eggs, obtained from Winter Egg Farm (UK), were incubated at 38°C in humidified atmosphere until required stages.

In utero electroporation

Transfection by electroporation of embryonic neural progenitors was performed as described previously (García-Moreno et al., 2010). Pregnant

mice were anaesthetised by inhalation with isoflurane administered in conjunction with pure oxygen. The uterine horns were exposed out of the abdominal wall and constantly warmed and hydrated with warm saline. Embryos were injected to specifically fill up the lateral ventricle. The embryos were then electroporated. Forebrain ventricular zone cells were transfected by means of a BTX Electroporator ECM (Harvard Apparatus). Buprenorphine (vetergesic) was administered to the pregnant mice prior to surgery (0.05 mg/kg) and the injected embryos were allowed to survive until E15 or E18 or until different postnatal stages.

In ovo electroporation

Electroporation of the chick embryos was performed as previously described (Itasaki et al., 1999). Briefly, eggs were incubated in vertical position at 38°C. Plasmids were injected into either the lateral ventricles or the wing limb bud of E4/E5 chick embryos using a fine pulled-glass needle. Four electric pulses (14–17 V, 15 ms pulses with a 950 ms interval) were then applied to the brain between insulated silver 40 mm×0.8 mm wire electrodes with flattened pole (Intracel). Drops of Ringer's solution supplemented with antibiotics (penicillin/streptomycin: Sigma) were added to the egg and the embryos were incubated until E7–E14.

Tissue processing

Embryonic murine brains, chick embryonic brains and wing limbs up to E11 were fixed by immersion in 4% paraformaldehyde (PFA), whereas postnatal mice and E12–E14 chick embryos were transcardially perfused with phosphate-buffered saline (PBS) followed by PFA. All the brains were coronally sectioned at 50–70 μm in a vibrating microtome (Leica VT1000S).

Imaging and analysis

Images were captured using a Zeiss LSM 710 confocal microscope (Carl Zeiss Microimaging). Similar image parameters (laser power, gain, pinhole and wavelengths) were maintained for images from each brain. To obtain a clear definition of the thinnest processes of the neurons, we needed to increase the laser power of the confocal microscope to a level in which the brightest nuclei could appear saturated in the final image. However, when using this laser power, we still detected a wide range of colour intensities and this makes the analysis of different cells possible. Three independent channels were employed to detect the four fluorophores, the first for EGFP, the second for mT-Sapphire and mCherry, and the third for EYFP. The excitation and absorption conditions for each fluorophore were (in nanometres): mT-Sapphire (Ex: 405; Ab: 470–545), EGFP (Ex: 488; Ab: 490–530), EYFP (Ex: 514; Ab: 515–580), mCherry (Ex: 561; Ab: 580–695). Each channel was assigned as the emission colour, except for mT-Sapphire, which was assigned as blue. Z-stacks were taken individually for each channel and then collapsed to obtain maximum intensity projections. Images were adjusted and analysed using ImageJ (Image Analysis in Java, NIH) and Adobe Photoshop CS6 (Adobe Systems Inc.).

To establish clonal relationships, we analysed the intensity mean of each fluorescent signal separately using IMARIS software (Bitplane; Switzerland) and classified intensities in six values (0 to 5). It generated a four-digit code easily comparable among cells. All the values were compared across potentially clonally related cells. In ideal conditions, two independent cellular compartments should be measured separately. In our experience, the nuclear labelling can be easily identified and measured independently. So for a better performance of CLoNe in neurons, of which the nucleus usually covers most of the soma, independent nuclear and membrane/cytoplasmic measurements are recommended.

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Competing interests

The authors declare no competing financial interests.

Author contributions

F.G.-M. and Z.M. designed the project; F.G.-M. performed the experiments; N.A.V. performed Tbr2^{Cre} electroporations; J.B. designed the chick experiments; F.G.-M., N.A.V. and Z.M. interpreted the results; F.G.-M. and Z.M. wrote the manuscript; all the authors commented and edited the manuscript.

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Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.105254/-/DC1>

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