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Lineage Tracing and Clonal Analysis in Developing Cerebral Cortex Using Mosaic Analysis with Double Markers (MADM)
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Corresponding Author:	Simon Hippenmeyer Institute of Science and Technology Austria Klosterneuburg, AUSTRIA
Corresponding Author's Institution:	Institute of Science and Technology Austria
Corresponding Author E-Mail:	simon.hippenmeyer@ist.ac.at
Order of Authors:	Robert Beattie
	Carmen Steicher
	Nicole Amberg
	Giselle Cheung
	Ximena Contreras
	Andi H. Hansen
	Simon Hippenmeyer
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IST AUSTRIA

Institute of Science and Technology

Simon Hippenmeyer
Professor

Phone +43(0)2243-9000-5101
simon.hippenmeyer@ist.ac.at
@HippenmeyerLab

Am Campus 1
3400 Klosterneuburg
Austria

Xiaoyan Cao, PhD,
Review Editor
Nam Nguyen, PhD,
Manager of Review JoVE
Ronald Myers, PhD,
Science Editor JoVE
1, Alewife Center, Suite 200
Cambridge, MA 02140
USA

Klosterneuburg, February 17, 2020

Dear Dr. Myers,
Dear Dr. Nguyen,
Dear Dr. Cao,

Please find enclosed our revised manuscript (JoVE61147R1) by Beattie et al., entitled *'Lineage Tracing and Clonal Analysis in the Developing Cerebral Cortex Using Mosaic Analysis with Double Markers (MADM)'* to be considered for publication in *JoVE*, and to be included in the methods collection focusing on cortical development (guest edited by Dr. Isabel Martinez Garay).

We have further addressed each of the editorial and peer review comments in our revision and used track changes to indicate all the edits and changes in the Word document. We also include in our submission a rebuttal document where we address all the points raised in the review process.

We hope that our revised manuscript is now suitable for publication and production of the video article.

Thank you very much for your time and consideration. We are looking forward to hearing from you soon.

Sincerely yours,

Simon Hippenmeyer, PhD

1 **TITLE:**
2 **Lineage Tracing and Clonal Analysis in Developing Cerebral Cortex Using Mosaic Analysis with**
3 **Double Markers (MADM)**
4
5 **AUTHORS AND AFFILIATIONS:**
6 Robert Beattie[1], Carmen Steicher[1], Nicole Amberg[1], Giselle Cheung[1], Ximena Contreras[1], Andi H.
7 Hansen[1], Simon Hippenmeyer[1]
8
9 [1]Institute of Science and Technology Austria, Klosterneuburg, Austria
10
11 **Corresponding Author:**
12 Simon Hippenmeyer   (simon.hippenmeyer@ist.ac.at)
13
14 **Email Addresses of Co-Authors:**
15 Robert Beattie   (robert.beattie@ist.ac.at)
16 Carmen Streicher   (carmen.streicher@ist.ac.at)
17 Nicole Amberg   (nicole.amberg@ist.ac.at)
18 Giselle Cheung   (giselle.cheung@ist.ac.at)
19 Ximena Contreras   (ximena.contreras@ist.ac.at)
20 Andi H. Hansen   (andi.hansen@ist.ac.at)
21

22 **KEYWORDS:**
23 mosaic analysis with double markers (MADM), clonal analysis, lineage analysis, stem cell, cerebral
24 cortex, radial glia progenitor (RGP), neurogenesis, neuroscience, neurodevelopment
25
26 **SUMMARY:**
27 A protocol to perform lineage tracing and functional genetic analysis of candidate genes at a
28 single cell level using mosaic analysis with double markers (MADM) is presented. MADM clonal
29 analysis provides a quantitative framework to measure the proliferative behavior, cellular
30 output, and lineage relationship of individual progenitors and their daughter cells.
31
32 **ABSTRACT:**
33 Beginning from a limited pool of progenitors, the mammalian cerebral cortex forms highly
34 organized functional neural circuits. However, the underlying cellular and molecular mechanisms
35 regulating lineage transitions of neural stem cells (NSCs) and eventual production of neurons and
36 glia in the developing neuroepithelium remains unclear. Methods to trace NSC division patterns
37 and map the lineage of clonally related cells have advanced dramatically recently. However,
38 many contemporary lineage tracing techniques suffer from the lack of cellular resolution of
39 progeny cell fate, which is essential for deciphering progenitor cell division patterns. Presented
40 is a protocol using mosaic analysis with double markers (MADM) to perform in vivo clonal
41 analysis. MADM concomitantly manipulates individual progenitor cells and visualizes precise
42 division patterns and lineage progression at unprecedented single cell resolution. MADM-based
43 interchromosomal recombination events during the G2-X phase of mitosis, together with
44 temporally inducible *CreER^{T2}*, provide exact information on the birth dates of clones and their

45 division patterns. Thus, MADM lineage tracing provides unprecedented qualitative and
46 quantitative optical readouts of the proliferation mode of stem cell progenitors at the single cell
47 level. MADM also allows for examination of the mechanisms and functional requirements of
48 candidate genes in NSC lineage progression. This method is unique in that comparative analysis
49 of control and mutant subclones can be performed in the same tissue environment in vivo. Here,
50 the protocol is described in detail, and experimental paradigms to employ MADM for clonal
51 analysis and lineage tracing in the developing cerebral cortex are demonstrated. Importantly, this
52 protocol can be adapted to perform MADM clonal analysis in any murine stem cell niche, as long
53 as the *CreER^T2* driver is present.

54
55 **INTRODUCTION:**
56 The cerebral cortex is a highly organized structure composed of six distinct layers. The cortex
57 contains a diverse array of cell types including neurons and glia, which interact to form functional
58 neural circuits. Most, if not all, cortical excitatory projection neurons and glia are derived from a
59 common pool of neural stem cells (NSCs) known as the radial glial progenitors (RGPs)[1-3]. RGPs
60 themselves are derived from neuroepithelial stem cells (NESCs) composing the early embryonic
61 neuroepithelium. By embryonic day 9 (E9) in mice, NESCs begin to transition into RGPs[4]. RGP
62 lineage progression requires precise temporal and spatial regulation, and when this process is
63 hindered, severe neurological disorders such as megalencephaly, microcephaly, lissencephaly, or
64 impairments such as schizophrenia and autism can result[5,6]. At E10, most RGPs undergo
65 symmetric proliferative divisions, resulting in an expansion of the neural progenitor pool[4,7]. RGPs
66 eventually begin to divide asymmetrically, producing cortical projection neurons in a temporally
67 defined manner. Through consecutive waves of neurogenesis, newborn neurons migrate into the
68 cortical plate forming cortical laminae with early born neurons occupying deep layers and late
69 born neurons residing in the superficial layers[8-10]. Because clonally related pyramidal neurons
70 migrate radially into the cortex with very little tangential dispersion, daughter cells tend to form
71 a column or cone shaped structure referred to as a neuronal radial unit[4,11-13]. By E17, embryonic
72 neurogenic expansion is complete in mice[14]. RGPs can also produce ependymal cells and some
73 classes of glia, including astrocytes and oligodendrocytes[1,15-19]. The potential of RGPs to give rise
74 to both neurons and astrocytes seems to be consistent across all cortical regions[18], with
75 approximately 1/6 of neurogenic RGPs also producing glia[11].

76
77 Currently, the genetic and epigenetic factors regulating temporal progression of a stem cell along
78 its lineage are mostly unknown. Temporal patterns of gene expression may have substantial
79 impact on lineage decisions in RGPs[20-24]. How this tightly knit relationship between temporal and
80 spatial patterning leads to the molecular diversity of adult neuronal types across cortical areas is
81 not known. Likewise, how the individual stem cell potential and its cellular output is modulated
82 at the cellular and molecular level is an important unanswered question. Future studies will
83 hopefully address some of these questions, ultimately expanding our understanding of functional
84 cortical circuit formation.

85
86 Developmental neurobiology seeks to understand the lineage relationship that cells in the brain
87 share with one another. Initially, very few research tools were available for this, and many early
88 studies relied on visual observations of division patterns in transparent organisms such as

89   *Caenorhabditis elegans*[25]. Recent decades have seen a dramatic increase in the number and
90   sophistication of techniques available[13,26-29]. The emergence of the CRISPR-Cas9 genome editing
91   system allows for synthetic reconstruction of cell lineage relationships by introducing evolving
92   DNA barcodes[27,30]. Two recent examples of barcoding strategies include the use of homing guide
93   RNA that directs CRISPR-Cas9 to specific DNA barcode loci or a cytidine deaminase fused with
94   nickase Cas9 to target endogenous interspersed repeat regions[31,32]. These technologies provide
95   highly multiplexed approaches through the introduction of barcodes that progressively and
96   stably accumulate unique mutations over time. Genome editing approaches are highly valuable
97   because they allow retroactive analysis of the relationship between any two cells based on the
98   shared inheritance of these barcodes. However, in order to read the barcodes in individual cells,
99   the tissue usually must be disrupted, and therefore information regarding position, morphology,
100   and absolute cell numbers from an individual progenitor is lost.
101
102   Combinatorial labeling paradigms preserve spatial information and in principle also allow for the
103   distinction between closely localized or even overlapping clones[33,34]. For a lineage tracing method
104   to be informative it must label individual progenitors and their progeny in a sparse and indelible
105   manner. Notably, the Brainbow[35] and Confetti[36,37] approaches use stochastic multicolor *Cre*
106   recombinase-based reporters that express a combination of fluorescent proteins from a single
107   locus. The extensive number of simultaneous color combinations that can be achieved in vivo
108   make this a powerful tool when tracing cortical RGP clones and astrocytes[34]. Transposon-based
109   systems providing stable genomic integration of transgenes encoding fluorescent reporters and
110   permitting lineage tracing of cortical progenitors have also been developed[33,38-41]. Transposon-
111   based systems have an added advantage in that the reporter constructs stably integrate into the
112   genome, and thereby reliably label lineally related daughter cells. To trace astrocyte lineages
113   specifically, a number of methods have been developed that involve electroporation of piggyBac
114   transposases including *Star Track*, which makes use of a combination of constructs encoding
115   different fluorescent proteins[40,42]. Another approach, *MAGIC markers*, introduces Brainbow
116   vectors as transposable transgenes. This has been successfully used to trace embryonic neural
117   and astrocyte progenitors[34,43]. Recently, mosaic analysis by dual recombinase-mediated cassette
118   exchange (MADR) was found to stably label mutant cells expressing transgenic elements from
119   precisely defined chromosomal loci[44]. These powerful in vivo combinatorial labeling techniques
120   have provided numerous insights into the lineage dynamics of progenitor cells. However, these
121   analyses are performed on fixed tissue, providing a snapshot of individual clones at a defined
122   developmental stage. In order to observe changes in the lineage dynamics of single clones over
123   time, chronic in vivo imaging methods similar to those performed in the adult dentate gyrus need
124   to be applied[45].
125
126   Mosaic analysis with double markers (MADM) is a powerful dual color labeling method that
127   enables in vivo lineage tracing of individual progenitor cells in mice[46,47]. Two components are
128   necessary for MADM labeling events to occur: First, MADM cassettes must be targeted to
129   identical loci on homologous chromosomes. Cassettes consist of two chimeric fluorescent
130   reporter genes, eGFP (green, [G]) and tandem dimer Tomato (red, tdT[T]). The *GT* cassette
131   contains the N-terminus of eGFP and the C-terminus of tdT, separated by an intron containing a
132   loxP site. The *TG* cassette is constructed inversely, with the N-terminus of tdT and the C-terminus

133 of eGFP. Second, the expression of *Cre* recombinase in the same cell containing the targeted
134 MADM cassettes is essential. In the absence of *Cre*, the chimeric cassettes do not express
135 functional eGFP or tdT because their coding sequences are disrupted. The loxP sites serve as the
136 target for Cre-mediated interchromosomal recombination, resulting in the reconstitution of both
137 expression cassettes simultaneously. If recombination occurs during the G2 phase of cell cycle
138 followed by X segregation (G2-X), the two daughter cells will each express one of the two
139 fluorescent proteins. Temporal regulation of *CreER^{T2}* activity using tamoxifen (TM) provides
140 precise information on birth date of MADM clones and the division patterns of their progeny
141 (**Figure 1A**)[29,46,47].
142
143 MADM can potentially systematically label individual clones with high single cell resolution in the
144 mouse brain similar to traditional but nonspecific and laborious methods like Golgi staining[48] or
145 dye filling[49]. Because only the promoter driving *CreER^{T2}* determines the cell type specificity of
146 clonal MADM labeling, MADM can in principle be applied for clonal lineage tracing throughout
147 any murine organ and tissue[47,50-52]. Indeed, studies have already used MADM to reveal lineage
148 relationships in clones derived from diverse tissues[47,50-59]. MADM experimental paradigms have
149 been applied to study lineage in cortical projection neurons, glia, and postnatal stem cells in the
150 developing neocortex[7,11,12,46,60-65]. MADM has also been used to study cell lineage in the adult
151 dentate gyrus, thalamus, cerebellar granule cells, and interneurons at the clonal level (see **Table
152 1** for a complete list)[47,53,54,56,57,66].
153
154 A unique feature of MADM is the ability to genetically link mutations distal to one MADM
155 cassette, thereby creating a genetic mosaic (**Figure 1B** and **Figure 2**). This results in wild type
156 daughter cells labeled with one fluorescent marker (tdT in **Figure 1B**) and homozygous mutant
157 siblings with the other (eGFP in **Figure 1B**) in an unlabeled heterozygous environment. MADM is
158 unique in that comparative analysis of control and mutant subclones can be performed in the
159 same tissue environment in vivo. Originally, MADM cassettes were targeted into the *Rosa26*
160 locus[47], but MADM analysis of gene function was limited to genes distal to the locus. To overcome
161 (at least partly) this limitation and expand the possibilities for MADM-based gene analyses,
162 MADM cassettes were knocked in close to the centromeres of Chr. 7[51], Chr. 11[46], and Chr. 12[51].
163 Targeting all 19 mouse autosomes with MADM cassettes is in progress and will allow for virtually
164 any gene to be studied in the future, providing an unparalleled platform for the study of
165 developmental lineage relationships in combination with functional genetic analysis.
166
167 **PROTOCOL:**
168
169 Mouse protocols were reviewed by institutional preclinical core facility (PCF) and internal ethical
170 committee at IST Austria. All breeding and experimentation were performed under a license
171 approved by the Austrian Federal Ministry of Science and Research in accordance with the
172 Austrian and EU animal laws.
173
174 **1. Breeding of experimental mice for MADM clonal analysis**
175
176 1.1. Set up timed experimental MADM matings (>P56; CD-1) in the late afternoon (5:00 PM) and

177   check for vaginal plugs the proceeding morning (8:00 AM). The morning the plug is present
178   counts as day 0.5. See **Figure 2** for an overview of the experimental mouse mating setup. Ensure
179   that timepoints for TM induction of *CreER^{T2}* activity and analysis are appropriate to address
180   experimental questions.
181
182   NOTE: For more information refer to **Figure 3** and representative results below.
183
184   1.2. For postnatal sampling, set up breedings to generate foster mothers in parallel.
185
186   NOTE: These should be started up to 1–2 days prior to setting up experimental breedings.
187
188   **2. TM induction in MADM mice**
189
190   2.1. Prepare a working 20 mg/mL TM solution by dissolving it in corn oil in a 15 mL or 50 mL
191   conical centrifuge tube and placing it on a rocking platform for ~4 h at room temperature (RT),
192   ensuring TM is completely dissolved. Store working solution at 4 °C covered with aluminum foil
193   and use within 2 weeks.
194
195   2.2. To induce MADM recombination events, deliver a single injection of TM intraperitoneally (IP)
196   using a 1 mL tuberculin syringe and a 25 G needle into a timed pregnant dam. Depending on the
197   stage of cortical neurogenesis, inject TM between E10–E15 at a dose of 1–2 mg/pregnant dam.
198   For early timepoints (i.e., E10) use a maximum of 1 mg/pregnant dam (25 mg/kg) to prevent
199   complications during pregnancy[11]. For timepoints between E11–E15 use 2 mg/pregnant dam (50
200   mg/kg)[7].
201
202   NOTE: Alternatively, TM can be administered with an oral gavage for late pregnancies.
203
204   2.3. For MADM clonal analysis to postnatal timepoints, recover live embryos at E18–E19 through
205   Caesarean section, and then raise pups with a foster mother.
206
207   NOTE: Depending on the health status of the pregnant female, it may not be necessary to
208   perform a Caesarean section but raising pups with a foster mother is still required because the
209   original TM-treated mother may have trouble lactating.
210
211   2.4. To recover live embryos by Caesarean section or to retrieve embryonic timepoints for
212   analysis, sacrifice the pregnant dam by cervical dislocation.
213
214   2.5. Place the animal in a supine position and disinfect fur with 70% ethanol. Make a small
215   incision in the skin in the lower abdomen above the uterus using surgical forceps and scissors.
216   Make a second incision through the muscles and the abdominal muscular wall to reveal the
217   peritoneum.
218
219   2.6. Remove the uterus by separating from the surrounding tissues with scissors. Transfer intact
220   uterus onto a glove with warm water to increase the embryo survival rate until each is removed

221   from the amnion individually.

223   2.7. Use fine tipped scissors and fingers to carefully open the uterine walls to release embryos.
224   Do not cut the umbilical cords too close to the body to prevent extensive blood loss. If embryos
225   are to be used for analysis, proceed to step 3.9. If pups are to be fostered, proceed to step 2.8.

227   2.8. If fostering is required, clean the pups before transferring them to the foster mother. While
228   cleaning the pups, gently press the chest from time to time to initiate breathing. Place back on a
229   second glove filled with warm water to improve survival rate.

231   NOTE: It is important to gently remove any remaining amnion and/or placenta with a paper
232   towel.

234   2.9. Before transferring pups to the foster mother, remove the foster mother from her cage,
235   remove the original pups, and replace with the experimental pups. Return the foster mother to
236   her cage.

238   NOTE: See discussion for additional suggestions to improve fostering acceptance rates.

240   2.10. If genotyping is required, collect toe or tail biopsies between P6–P8.

242   NOTE: Perform this step only if animal experimental licenses approve this practice.

244   **3. Tissue preparation for MADM clones in the brain**

246   NOTE: For experiments that include postnatal tissue (≥P4), proceed to step 3.1. For embryonic
247   timepoints and early postnatal (P0–P3), continue to step 3.9.

249   3.1. Anesthetize the experimental MADM animal with an IP injection of a
250   ketamine/xylazine/acepromazine solution (65 mg, 13 mg, and 2 mg/kg body weight, respectively)
251   and confirm that the mouse is unresponsive by pinching the paw.

253   NOTE: Both male and female MADM mice (CD-1 background) are used for analysis. If genotyping
254   is required, collect ear biopsies at this point.

256   3.2. Place the anesthetized animal in the supine position on the perfusion surgery tray and
257   disinfect fur with 70% ethanol. To begin surgery, carefully make an incision with scissors and
258   surgical forceps through the outer layer of the skin and then a second incision through the muscle
259   layer. Lift the tip of the sternum and cut connective tissue on the sides, taking extra caution to
260   avoid cutting the liver. The thoracic cavity will be visible.

262   3.3. Snip the diaphragm and lift to reveal the heart. Carefully trim the rib cage and pin to the
263   surgical tray to expose the heart. For pups, remove the rib cage completely.
264

265 3.4. Insert a needle with phosphate buffered saline (PBS) into the lower left ventricle (paler
266 tissue). Using small iris scissors make an incision to the posterior end of the right atrium (dark
267 red tissue) for the blood to drain.

268

269 3.5. Perform perfusion with PBS followed immediately by freshly made, ice-cold 4%
270 paraformaldehyde (PFA) prepared in PBS. For pups (P4–P10) use syringes to perform perfusion.
271 Fill one syringe with 10 mL of PBS and another with 10 mL of 4% PFA. Ensure all air bubbles in the
272 syringes have been removed. For older animals, use a peristaltic pump.

273

274 3.6. Begin perfusing with PBS (10 mL at 2–4 mL/min in pups; 20 mL at 4–6 mL/min for adults using
275 a peristaltic pump). The liver will become clear and pale yellow if the needle is positioned
276 correctly.

277

278 3.7. Once complete, remove the needle from pups and insert the needle containing PFA into the
279 same hole. For adults, stop the peristaltic pump before exchanging the PBS solution with ice-cold
280 PFA, making sure to avoid bubbles in the uptake tubing. Resume perfusing with PFA (10 mL at
281 2–4 mL/min in pups; 30 mL at 4–6 mL/min for adults using a peristaltic pump).

282

283 3.8. When perfusion is complete, decapitate the mouse and remove the brain through careful
284 dissection. Transfer brain to 4% PFA. Use at least 5x the brain volumes (i.e., ~5–10 mL of PFA in
285 a 15 mL conical centrifuge tube) and incubate overnight at 4 °C for postperfusion fixation to
286 ensure complete fixation of tissue. Continue to step 3.10.

287

288 3.9. For embryonic tissue and early postnatal tissue (i.e., P0–P3), after performing a Caesarian
289 section, decapitate the embryos with scissors. If genotyping is required, collect the tail of the
290 embryo at this point. Immediately dissect out the brain and transfer to a 12 well plate containing
291 2–3 mL of 4% PFA/well. Incubate overnight at 4 °C for postfixation.

292

293 3.10. Next morning exchange PFA with 10 mL (adult) or 2–3 mL (embryo) of PBS and repeat wash
294 3x for 15 min at RT. Transfer tissue to 30% sucrose solution in phosphate buffer (PB) and store at
295 4 °C on a rocking platform until the tissue sinks in the solution.

296

297 3.11. Embed the brain in optimal cutting temperature (OCT) compound in an embedding mold,
298 taking care to orient the brain for either coronal or sagittal sectioning. Freeze by placing the
299 embedding mold on dry ice until OCT becomes completely opaque (~10–15 min). Store tissue at
300 -80 °C until further use.

301

302 **4. Preparation of MADM tissue for immunohistochemistry**

303

304 4.1. Attach the tissue block to the specimen disk in the cryostat by applying a ring of OCT to the
305 disk and placing the block directly into the OCT when it begins to freeze. Ensure the block is
306 correctly oriented for the desired cutting plane.

307

308 NOTE: Here, coronal sectioning to investigate cortical MADM clones is described in detail.

309

310   4.2. Set the block temperature in the cryostat to -20 °C and the blade temperature to -21 °C.

311

312   4.3. Allow the tissue block to adjust to the chamber temperature by mounting the specimen disk
313   to the specimen holder and leave in cryostat for ~5 min before beginning sectioning.

314

315   4.4. Trim block in thick sections (45–60 µm) until the tissue region of interest is reached.

316

317   4.5. Once the edge of the cortex is clearly visible, stop sectioning and lock the blade. Ensure the
318   blade is shielded before trimming the block.

319

320   4.6. Trim excess OCT surrounding the tissue with a blade, leaving ~1–2 mm of OCT on all sides of
321   the brain.

322

323   4.7. Next orient the block so that one of the lateral edges of the cortex is oriented downward and
324   the other upwards (i.e., the most rostral edge of the cortex is pointed right).

325

326   4.8. Begin sectioning with a thickness of 45 µm for adult clones and 30 µm for embryonic clones.
327   Perform each section individually and use a small brush to keep the area below the knife clean
328   of any debris left while trimming the block.

329

330   NOTE: If this is not done and a section falls, it may be difficult to determine the correct order of
331   the slices.

332

333   4.9. If sections begin to curl, trim the edges of the block and/or carefully adjust the glass antiroll
334   plate.

335

336   4.10. For embryonic clone analysis, mount sections directly to a frosted slide. Dry on a heating
337   plate at 37 °C before proceeding directly to step 5.6.

338

339   NOTE: Several sections can be added to one slide, but ensure the sequential order is maintained.

340

341   4.11. To collect adult clones, prepare 24 well plates containing 1 mL of PBS/well (typically, 5–6
342   plates per brain). Starting from the first well, with cold forceps collect individual serial sections in
343   PBS in the order of sectioning.

344

345   NOTE: The floating section method is adopted for adult tissue to ensure that no sections are
346   missed and that mounted sections contain no wrinkles.

347

348   4.12. Stop sectioning once the end of the neocortex is reached.

349

350   4.13. For adult clones, proceed to mounting floating sections.

351

352   NOTE: Sections can be kept in PBS at 4 °C for up to 24 h.

5. Mounting adult tissue for imaging

NOTE: The following tools are required: small paint brush, Petri dish, PBS with 0.5% Tween (PBS-T), adhesion slides (**Table of Materials**), mounting medium (**Table of Materials**), coverslips, and forceps.

5.1. Fill a Petri dish with PBS-T.

NOTE: Detergent is used to aid in the mounting process. If staining for additional antigens that are sensitive to detergents (i.e., glycoproteins) is necessary, it is best to skip the addition of Tween.

5.2. Place an adhesion slide into the PBS-T so that it is nearly covered up to the label.

5.3. Transfer the first section into the PBS-T.

5.4. Using a small paint brush, maneuver the section onto the slide and arrange it to preserve the order of cutting. Proceed in the same way with all further sections.

5.5. Once all sections are in position, place the slide (~12–16 sections/slide) in a dark slide chamber. Slightly lift the lid to allow the sections to dry completely (~10–20 min), ensuring that they remain adhered in subsequent steps.

5.6. If performing immunohistochemistry for additional antigens, proceed directly to section 6 or 7.

NOTE: For embryonic timepoints, it is necessary to perform immunostaining steps for at least GFP and tdT (section 6). For adult clones, this is only required if staining for additional antigens in parallel (sections 6 and 7).

5.7. Rehydrate and wash sections 1x with 1x PBS for 5 min to remove residual PBS-T. Apply ~1 mL of 4',6-diamidino-2-phenylindole (DAPI) diluted in 1x PBS (1:5,000) to the slide, ensuring all sections are covered and incubate for 15 min.

5.8. Carefully remove the DAPI and wash 1x with 1x PBS for 5 min. Remove excess PBS and dry for ~1–2 min before embedding in 110 µL of mounting medium. Seal with a 24 x 60 mm coverslip and let dry for at least 3 h before imaging.

6. Immunostaining for GFP and tdT only

NOTE: This section is necessary for embryonic clones.

6.1. Place slides horizontally in a humidified slide incubation chamber. Mark slide boundaries

397   with a wax marker to minimize the amount of buffer required.
398
399   6.2. Rehydrate sections with 1x PBS. To improve staining quality, work with freshly sectioned
400   tissue.
401
402   6.3. Add 250–400 µL of blocking buffer (0.5% Triton X-100, 2–3% normal donkey serum in 1x PBS)
403   per slide, ensuring all sections are covered. Incubate for 1 h.
404
405   NOTE: The concentration of detergent (Triton X-100 or Tween-20) will vary depending on the
406   additional primary antibodies used because some antigens are more sensitive to detergents than
407   others.
408
409   6.4. Remove blocking buffer and add primary antibodies in blocking buffer to the slide (300–400
410   µL/slide).
411
412   NOTE: An example of a standard primary antibody reaction for anti-GFP/anti-tdT (MADM) could
413   use chicken anti-GFP (1:500) and rabbit anti-RFP (1:500).
414
415   6.5. Incubate with primary antibodies overnight at 4 °C.
416
417   NOTE: The slides must be incubated perfectly horizontally with buffer covering all sections.
418   Otherwise, uneven or poor staining may result.
419
420   6.6. Confirm the next morning that the blocking buffer with primary antibodies still covers all
421   sections on the slide. If not, repeat the incubation step for 3–4 h at RT.
422
423   6.7. Remove primary antibodies and wash 4x with 1x PBS for 10 min at RT.
424
425   6.8. Add secondary antibodies diluted in blocking buffer to slide (300–400 µL/slide): Alexa Fluor
426   488 anti-chicken IgG (1:500) and Cy3 anti-rabbit IgG (1:500).
427
428   6.9. Incubate at RT for 2 h. Keep slides covered from light to prevent bleaching of fluorophores.
429
430   6.10. Remove secondary antibodies and wash 2x with 1x PBS for 10 min.
431
432   6.11. Incubate with DAPI diluted in PBS (1:5,000) for 15 min.
433
434   6.12. Wash sections 1x with 1x PBS for 10 min.
435
436   6.13. Remove excess PBS and dry for ~1–2 min before embedding in 110 µL of mounting medium.
437
438   6.14. Seal with 24 x 60 mm coverslip and let dry for at least 3 h before imaging. Image slides
439   within 1–2 weeks after performing immunohistochemistry to ensure optimal signal.
440

7. Immunostaining for GFP, tdT, and additional antigens

7.1. Perform steps 6.1–6.3.

7.2. Remove blocking buffer and add primary antibodies in blocking buffer to the slide (300–400 µL/slide).

NOTE: When staining for three or more antigens (i.e., GFP, tdT, and a protein of interest) and the antibody for the protein of interest was raised in rabbit, it is recommended to use the anti-tdT (goat) primary antibody at a dilution of 1:500. An example of a primary antibody reaction for three antigens with alternative tdT staining could use chicken anti-GFP (1:500), goat anti-tdT (1:500), and antibody against the protein of interest (i.e., rabbit).

7.3. Perform steps 6.5–6.7.

7.4. Add a secondary antibody mix diluted in blocking buffer to slide (300–400 µL/slide): Alexa Fluor 488 anti-chicken IgG (1:500), Cy3 anti-goat IgG (1:500), and Alexa Fluor 647 anti-rabbit IgG (1:500).

7.5. Perform steps 6.9–6.14.

8. Confocal image acquisition and quantification of MADM clones

8.1. Identify and document brain sections containing clones and their locations in the cortex.

NOTE: The number of sections a clone spans will vary depending on when the clone was induced, the *CreER^{T2}* driver, and the time of analysis. This step can be performed on either a confocal microscope or an epifluorescence microscope.

8.2. Using an inverted confocal microscope, begin by selecting and configuring the correct laser lines and filters. For MADM brains, select DAPI, GFP, and tdT (excitation: 358 nm, 488 nm, and 554 nm, respectively; peak emission: 461 nm, 507 nm, and 581 nm, respectively). Ensure the pinhole is set to 1 airy unit for optimal imaging quality.

8.3. For confocal specific settings, image clones with a 20x objective and 1x zoom. For images that will be used in quantifications, use a scanning speed pixel dwell value of 1.52–2.06 µs (values 7–8 in the image acquisition software) with no averaging. Adjust laser intensity and gain settings for each channel as appropriate.

NOTE: Depending on the required image quality, settings for scanning speed and averaging may vary.

8.4. Once the clone is clearly identified, arrange imaging tiles to cover all relevant sections in the clone. Adjust the z-stack so that all MADM labeled cells in the clone are captured with an interval

485 <mark>of 1.5 µm/z-stack slice. Adjust the tiled region so that the entire width of the cortex is captured</mark>
486 <mark>when imaging the clone (i.e., from pial surface to the corpus callosum).</mark>

487

488 <mark>8.5. Image individual clones spanning multiple sections consecutively, ensuring that any sections</mark>
489 <mark>without cells within a clone are still imaged for the purpose of 3D reconstruction and correct</mark>
490 <mark>interpretation of cell spatial information.</mark>

491

492 <mark>8.6. Analyze each section containing cells of a MADM clone sequentially from the rostral to the</mark>
493 <mark>caudal end of the cortex. Distinguish individual neurons and glia based on their morphology</mark>
494 <mark>and/or marker staining. Record positional information in parallel based on respective layer</mark>
495 <mark>boundaries defined by nuclear staining (DAPI).</mark>

496

497 NOTE: See **Figure 4** for representative results for embryonic analysis and **Figure 5** for
498 representative results for adult analysis.

499

500 <mark>**9. Serial 3D reconstruction of clones**</mark>

501

502 NOTE: The 3D reconstruction of individual clones imaged over serial brain sections is useful for
503 visual display as well as the analysis of 3D clonal architectures and can be performed according
504 to the following steps.

505

506 <mark>9.1. Stitch and fuse confocal tiled images based on acquisition parameters using image</mark>
507 <mark>acquisition software. Open .czi file and then run the **Stitching** method under the **Processing** tab.</mark>

508

509 <mark>9.2. Export stitched image stacks as individual z-planes in TIFF format. Open stitched .czi file and</mark>
510 <mark>then run the **Image Export** method under the **Processing** tab. For multichannel images, export as</mark>
511 <mark>Red/Green/Blue images for subsequent image processing.</mark>

512

513 9.3. Repeat steps 9.1 and 9.2 for each serial brain section of a clone.

514

515 NOTE: For accurate 3D reconstruction, all brain sections within a clone, including the ones
516 without labeled cells, must also be processed.

517

518 9.4. <mark>Concatenate individual images into a single stack in order beginning from the most rostral to</mark>
519 <mark>the most caudal z-plane using open source image processing software such as ImageJ/Fiji[67,68].</mark>

520

521 NOTE: Any blank images at the edges of each brain section should be removed at this point.

522

523 9.5. If required, correct the image stack obtained from step 9.4 for misalignment using an ImageJ
524 plugin called "**MultiStackReg**" by following steps 9.5.1–9.5.5. If image alignment is not required,
525 proceed to step 9.6.

526

527 NOTE: This plugin performs image alignment of the channel with highest contrast (usually DAPI)
528 and then applies the recorded transformation to the other channels, thus allowing reliable image

529 alignment of multichannel stacks. An auxiliary plugin called "**TurboReg**" must be preinstalled.
530
531 9.5.1. In ImageJ, install the "**MultiStackReg**" and "**TurboReg**" plugins.
532
533 9.5.2. Open the image stack of clone images obtained from step 9.4 to be aligned. Split channels
534 into DAPI (blue), GFP (green), and tdT (red) within **Color** option under **Image** tab.
535
536 9.5.3. Run "**MultiStackReg**" plugin to align DAPI channel by "**Rigid Body**" transformation and save
537 the transformation file.
538
539 9.5.4. Apply the saved transformation file to the other two channels using "**MultiStackReg**".
540
541 9.5.5. Merge all three aligned channels and save aligned stack.
542
543 9.6. To orient the clone in ImageJ rotate stack of clone images obtained from step 9.4 (or step
544 9.5.5 after alignment) in a vertical orientation with the pial surface on top and the corpus
545 callosum at the bottom. Crop in the xy plane if necessary.
546
547 9.7. For both qualitative presentation and quantitative analysis, generate a maximum z-
548 projection image (step 9.8) or perform 3D rendering (step 9.9) of the clone.
549
550 9.8. In ImageJ, open the image stack from step 9.6 and select **Z-projection** option with projection
551 type **Max intensity**. This will generate an image of the entire clone projected on the same plane.
552
553 9.9. In ImageJ, open the image stack from step 9.6 and select **3D Project** z-function to generate
554 a 3D visualization of the clone that can be rotated.
555
556 NOTE: It is important in this step to input the correct slice interval equivalent to the thickness of
557 individual z-stacks during image acquisition. The interpolation tool should be used to remove
558 gaps between slices.
559
560 **REPRESENTATIVE RESULTS:**
561 MADM results in the reconstitution of functional green and red fluorescent proteins with two
562 daughter cells each expressing one of the two fluorescent proteins upon G2-X chromosome
563 segregation events (**Figure 1A**). Because MADM events result in permanent and distinct labeling
564 of the two descendent lineages, quantifiable assessment of green and red daughter cell lineages
565 (subclones) can be performed. Variables including division pattern (e.g., symmetric versus
566 asymmetric) and potential (e.g., the number of progeny) of the original progenitor can be
567 determined. One daughter cell may exit the cell cycle, only producing one daughter cell, or
568 continue dividing to produce more neurons. Quantifying each fluorescently labeled subclone is
569 informative when retroactively determining if the original progenitor cell is undergoing
570 symmetric proliferative divisions, or asymmetric, neurogenic divisions at the time of TM
571 induction. Previous studies grouped *Emx1-CreER^{T2}* or *Nestin-CreER^{T2}* derived excitatory
572 projection clones in the cortex into two broad classes[7,11,46]. The first, termed "symmetric

573 proliferative clones", are composed on average of a considerable number of neurons, with both
574 green and red subclones containing four or more neurons each. The second group, "asymmetric
575 clones" defines a class of clones where the "minority" subclone contains fewer than three
576 neurons and the "majority" subclone, four or more[11]. These definitions are specific to cortical
577 RGPs and may need to be revisited for other brain regions and tissues. For both classes of cortical
578 clones, progeny will be distributed throughout the superficial and deep layers.
579
580 When designing MADM clonal studies there are a number of aspects that must be taken into
581 consideration. The time when MADM events are induced by administration of TM is a key
582 consideration (**Figure 3**). For cortical excitatory projection neuron MADM clones (i.e., using
583 *Emx1-CreER[T2]* or *Nestin-CreER[T2]*) at E10, nearly all RGPs were still undergoing symmetric
584 divisions[11]. Therefore, induction at E10 with TM captured multiple rounds of proliferative RGP
585 amplification and resulted in clones with high neuron numbers. However, the number of RGPs at
586 E10 was generally small and thus TM administration generated very few MADM events
587 (sometimes less than one per brain). The majority of RGPs switched from symmetric to
588 asymmetric neurogenic divisions at around E12. To target strictly asymmetric neurogenic clones,
589 it was best to induce at E12 or later (**Figure 3**). The time between TM induction and observing
590 MADM recombination events in the cortex tended to be less than 24 h. IP injections were the
591 preferred method for administering TM at embryonic stages for this method because it led to
592 greater reproducibility in clonal induction. It is also important to keep the TM dose to a minimum
593 for two reasons. First, if the MADM recombination rate increases, the probability of inducing
594 multiple, perhaps overlapping, clones is higher. Second, if too much TM is delivered, an increased
595 rate of abortion, embryo reabsorption, and smaller litter sizes may be observed. Abortions in
596 approximately half of all pregnant dams was observed when TM injections were delivered at E10.
597 This frequency decreased from E11 onwards and diminished to approximately 1/3 of pregnant
598 dams aborting. For a summary of TM doses, induction times, and *CreER[T2]* drivers used in previous
599 MADM studies, refer to **Table 1**. Reporter activity in the absence of TM was observed with some
600 TM-inducible *CreER[T2]* drivers[69]. Ectopic expression or MADM recombination events in the
601 absence of TM was not observed with the *Emx1-CreER[T2]* of *Nestin-CreER[T2]* drivers. This may be
602 partially due to the fact that TM-mediated chromosomal trans-recombinations occur at
603 approximately 1:1,000 to 1:10,000 lower frequency than cis-recombinations, reducing the
604 probability of ectopic MADM labeling.
605
606 Another factor to consider when planning a MADM clonal analysis experiment is the duration of
607 the study. Varying the length of the time between TM induction and when the experiment was
608 analyzed (A) (time window) displays stem cell dynamics over time[64]. Short embryonic time
609 windows (i.e., TM/E11–A/E13; TM/E11–A/E16) captured the dynamics of embryonic
610 neurogenesis (**Figure 4**). Comparing clones from two or more time windows provides quantitative
611 insight into the number of cells produced and how neuron distribution varies at different stages
612 of lineage progression[64]. To capture the entire potential of individual clones, it is necessary to
613 extend the time window analyzed into postnatal or adult timepoints[7,11,12]. Examples of
614 neocortical clones induced in the embryo and analyzed in the adult are shown in **Figure 5**. Of
615 note, cortical neurogenesis is mostly completed and gliogenesis increases by E17. Approximately
616 1/6 neurogenic RGP also proceed to generate astrocytes and/or oligodendrocytes[11].

617
618   Symmetric clones occur when RGPs undergo one or more rounds of proliferative division[11]. RGP
619   clones induced between E10–E12 were on average larger in size and provided more spatial
620   features of the final neuron distribution (**Figure 4A-C**). Clones with neurons relatively equally
621   distributed throughout deep and superficial layers took on a "cylinder" shape while clones with
622   neurons more dispersed in superficial layers than deeper layers developed a "cone" shape[11]. To
623   fully capture the spatial and morphological information of a clone, it was necessary to
624   computationally reconstruct each clone using sequential images. To measure clonal dispersion,
625   the maximal lateral dispersion (measured in all dimensions) in superficial layers (LII–VI) of a clone
626   was compared to neuron dispersion in deep layers (LV/LIV). This ratio (distribution
627   upper:distribution lower) provided a quantifiable readout of the overall clone shape.
628
629   Asymmetric clones, where the minority subclone was three or less, provided insight into the
630   neuronal output of a single RGP (**Figure 4D-F** and **Figure 5A-F**)[7,11,12]. The majority population
631   (large subclone) could be labeled either in red or green, with an average of approximately seven
632   excitatory projection neurons per clone when induced using an *Emx1-CreER*[T2] or *Nestin-CreER*[T2]
633   (**Figure 5G**)[7,11,12]. The total number of cells in a MADM clone could be further dissected by
634   analyzing the distribution of neurons in the large subclone across superficial and deep layers. The
635   minority population (small subclone) was labeled by the reciprocal color and was on average 1–2
636   cells per clone (**Figure 5H**). The total "unit size", which was on average 8–9 neurons, could be
637   calculated by adding the small and large subclones together (**Figure 5I**)[7,11,12]. It is important to
638   note that while the neuronal output of RGPs was highly predictable, there was a degree of clonal
639   heterogeneity[12,70].
640
641   Introduction of a mutation distal to the MADM cassette enables the generation of genetic
642   mosaics, providing a unique method to dissect the molecular regulators of stem cell lineage
643   progression. As such, MADM provides an unparalleled experimental platform to study the cell-
644   autonomous function of a gene (e.g., its association to microcephaly or macrocephaly). By
645   comparing clones induced in a MADM genetic mosaic to clones induced in a control MADM, a
646   highly quantitative readout of changes in neuron numbers and distribution can be generated.
647   Previous MADM-based studies quantified the cell-autonomous function of *Otx1* in microcephaly
648   formation at the clonal level (see **Figure 6A-E** for a representative example)[11]. In another study,
649   MADM clonal analysis demonstrated that *Ndel1* does not cell-autonomously regulate projection
650   neuron numbers, but instead the ability of newborn neurons to enter or migrate within the
651   cortical plate, which later forms the adult cortex[46]. These studies demonstrated the highly
652   quantitative nature of MADM clonal analysis in studying the cell-autonomous functions of genes
653   regulating cortical development. There are currently no examples in the literature using MADM
654   to study genes implicated in macrocephaly at the clonal level. However, in future studies analysis
655   of genes relevant to the control of cortical size in general can provide highly desirable insights at
656   the molecular and cellular level.
657
658   **FIGURE AND TABLE LEGENDS:**
659
660   **Figure 1: The MADM principle for lineage tracing and clonal analysis at single stem cell level.**

661 (**A**) To perform lineage tracing and clonal analysis with MADM, two components must be present.
662 First, MADM cassettes must be targeted to identical loci on homologous chromosomes. Cassettes
663 consist of two chimeric fluorescent reporter genes, eGFP (green, [G]) and tandem dimer Tomato
664 (red, tdT[T]). The *GT* cassette contains the N-terminus of eGFP and the C-terminus of tdT,
665 separated by an intron containing a loxP site. The *TG* cassette is constructed inversely, with the
666 N-terminus of tdT and the C-terminus of eGFP. Second, the expression of *Cre* recombinase must
667 occur in the cell containing the targeted MADM cassettes. The loxP sites serve as a target for Cre-
668 mediated interchromosomal recombination, resulting in the reconstitution of both expression
669 cassettes simultaneously. If recombination occurs during the G2 phase of the cell cycle followed
670 by X segregation (G2-X), the two daughter cells will express one of the two fluorescent proteins.
671 (**B**) MADM principle for genetic mosaic analysis at a single clone level. Mutant alleles (point
672 mutations, deletions, insertions, loxP-flanked conditional alleles as depicted in **Figure 1B**, etc.)
673 can be introduced distal to the TG-MADM cassette via meiotic recombination (see **Figure 2** and
674 Hippenmeyer et al.[46] for details on how to introduce mutant alleles into the MADM system). If a
675 G2-X Cre recombinase-mediated interchromosomal trans-recombination occurs between MADM
676 cassettes it results in one GFP+ homozygous mutant cell (*GeneX^{-/-}*) for the gene of interest and
677 one tdT+ homozygous wild type cell (*GeneX^{+/+}*) in an unlabeled heterozygous environment[46,47,71].
678 Alternate labeling outcomes not used in the clonal analysis (i.e., yellow cells) have been
679 previously described in detail[11,46,47].
680
681 **Figure 2: Breeding schemes for generation of experimental MADM mice for lineage tracing.**
682 Breeding scheme for the generation of control MADM (**A**) and *Gene X* MADM (**B**) experimental
683 MADM mice for clonal analysis. For more information regarding MADM breeding paradigms see
684 Beattie et al.[7] and Hippenmeyer et al.[7,46].
685
686 **Figure 3: Time course paradigms for MADM-based clonal lineage analysis.** Schematic of the
687 experimental design time windows. For longitudinal sampling paradigms, the timepoint of clone
688 induction remained constant and the length of time before analysis varied. In progressive interval
689 sampling, the timepoint of analysis remained constant, but the time of induction varied. A
690 combination of one or both approaches can be used depending on the questions addressed.
691
692 **Figure 4: MADM clonal analysis in the developing and adult neocortex.** TM-mediated MADM
693 clone induction in symmetrically proliferative (TM at E10) (**A**–**C**) and asymmetrically neurogenic
694 (TM at E12) (**D**–**F**) dividing RGPs. Depicted are individual MADM clones in vivo in the developing
695 (TM/E10–A/E16 and TM/E12–A/E16) (**B,E**) and adult (TM/E10–A/P21 and TM/E12–A/P21) (**C,F**)
696 in *MADM-11[GT/TG]*; *Nestin-CreER[T2+/-]* (**B,E**) and *MADM-11[GT/TG]*; *Emx1-CreER[T2+/-]* (**C,F**). Neuron
697 output was independent of subclone color and green majority/minority subclones could be
698 compared to red majority/minority subclones under control conditions[7,11]. Approximately 1/6 of
699 adult clones also contained astrocytes and/or oligodendrocytes, indicated by white asterisks.
700 Panels **B** and **F** are reproduced with permission from Hippenmeyer et al.[46] and Rulands and
701 Simons[72], respectively. CP = Cortical plate.
702
703 **Figure 5: MADM clonal analysis to quantify RGP-mediated neuron output.** Analysis of excitatory
704 neuron (unit) production by individual neurogenic RGPs at the clonal level using MADM[7,11]. (**A**)

705   Experimental paradigm for inducing mostly asymmetric MADM clones in the developing cortex.
706   (**B**) Possible asymmetric clone outcomes with the majority subclone labeled in either green or
707   red (**C**) Representative consecutive sections spanning a single neurogenic asymmetric clone (**D,E**)
708   3D reconstruction images of representative asymmetric G2-X MADM clones with majority
709   population in red (**D**) or green (**E**) in *MADM-11$^{GT/TG}$*; *Emx1-CreER$^{T2+/-}$* with TM induction at E12
710   and analysis at P21. Note both green and red labeled cells are wild type. (**F**) Schematic indicating
711   the two possible experimental MADM clone outcomes. (**G**) Quantification of the size of the
712   majority population arising from renewing RGPs in MADM-11 clones. (**H**) Quantification of the
713   size of the minority population arising from renewing RGPs in MADM-11 clones. (**I**) Quantification
714   of the unitary size of asymmetric neurogenic MADM-11 clones. Hypothetical values could
715   represent mean ± SEM. Scale bar = 100 μm (**D** and **E**). TM = Tamoxifen.
716
717   **Figure 6: MADM clonal analysis to study genes that lead to microcephaly and macrocephaly.**
718   Hypothetical MADM clonal analysis results when performing functional genetic dissection of
719   candidate genes that lead to microcephaly or macrocephaly. To dissect the cell-autonomous
720   functions of a gene of interest (*Gene X*) on neuron output, MADM requires mutant alleles to be
721   introduced distal to the MADM cassettes via meiotic recombination (for details how to introduce
722   mutant alleles into the MADM system see also **Figure 2**, Hippenmeyer et al.[46], and Laukoter et
723   al.[46,73]). (**A,B**) Schematic indicating experimental MADM paradigm for functional analysis of clonal
724   RGP units. The mutant subclone can either form the minority (**A**) or majority (**B**) population. (**C–E**)
725   Hypothetical MADM clonal analysis results when quantifying control MADM (white bars), *Gene-*
726   *X* MADM microcephaly (gray bars) and *Gene-X* MADM macrocephaly black bars) asymmetric
727   clones. (**C**) Quantification of the size of the majority population. (**D**) Quantification of the size of
728   the minority population. (**E**) Quantification of the unitary size of asymmetric neurogenic clones.
729   Hypothetical values could represent mean ± SEM. S = Hypothetical scenario where difference in
730   subclone cell number could reach significance, relative to control.
731
732   **Table 1: MADM clonal studies in the literature.** Summary of studies in the literature containing
733   MADM clonal lineage experiments, including *CreER^{T2}* driver used, TM dose, and time of injection.
734
735   **DISCUSSION:**
736   A method to use MADM to track cell lineage of individual RGPs in vivo in the developing
737   neocortex is described. When combined with TM-inducible *CreER^{T2}*, MADM events can be
738   precisely timed, providing a highly qualitative and quantitative visual readout of stem cell division
739   patterns at the single cell level. By titrating the dose of TM delivered, in an ideal situation an
740   average of less than one clone per cortical hemisphere can be obtained, providing adequate
741   spatial separation to unambiguously distinguish individual clones. By maintaining tissue integrity,
742   this method also captures essential information regarding position, morphology, and absolute
743   cell numbers. MADM cassettes on Chr. 11[7,11,12,46,56,57], on Chr. 7[51], and the original MADM at
744   *Rosa26*[47,53,59] have been used in MADM clonal analysis studies. The high-resolution of individual
745   cells provides unprecedented insight into both morphology and the clonal relationship of
746   daughter cells and permits the live imaging of proliferating stem cells and emerging clones[46,52].
747
748   Caesarean section and fostering of pups for analysis of clones at postnatal timepoints is a

749 necessary and critical step in the protocol. Depending on the health status of the TM-treated
750 pregnant dam, it may not be necessary to perform a Caesarean section. However, raising the
751 pups with a foster mother is still required, because the TM-treated mother may have trouble
752 lactating. No differences have been observed in the need for fostering with different *CreER^{T2}*
753 drivers. Both MADM lines and foster mothers are maintained on an outbred CD-1 background. If
754 Caesarean section is not necessary, the TM-treated pregnant dam used to generate experimental
755 pups may be reused for additional experimental breedings in accordance to principles of 3R (note
756 that this can only be done if animal experimental licenses approve of this practice). Foster
757 mothers can be used for fostering pups within 2 days after they give birth, but higher success
758 rates have been observed when foster mothers give birth on the same day as the experimental
759 mice that must be fostered. Therefore, it is important to set up timed matings for foster mothers
760 in parallel to experimental matings in step 1.1. Maintaining a similar litter number as the original
761 foster mother's litter can improve the survival rate of fostered pups, and therefore removal of
762 some to all of the original litter may be necessary. Additional steps that may improve fostering
763 includes rubbing the experimenter's gloves with litter and food (to remove the scent of the
764 gloves); rubbing the pups gently after the Caesarean section with fragments of the foster
765 mother's soiled litter and nest; and placement of the pups in close contact with the foster
766 mother's pups prior to their placement in the foster mouse cage.
767
768 As in other reporter-based lineage tracing methods, careful consideration must be taken when
769 choosing the optimal *CreER^{T2}* driver for MADM clonal experiments. First, the promoter used must
770 express the recombinase both temporally and spatially in the progenitor population of interest.
771 Finding this promoter can be challenging, because some promoters may change expression
772 patterns or become silenced at different stages of development. To improve cell type specificity
773 multiple site-specific recombinases, each driven by separate promoters, have been used. When
774 one or both recombinases are expressed in the same cell, this labels the cell and its progeny with
775 a fluorescent reporter[74-77]. In summary, it is important to choose a *CreER^{T2}* driver that is specific
776 to the population of progenitors being analyzed.
777
778 The most critical step in this method is the identification of a clone, because all cells must be
779 derived unambiguously from a single recombination event (step 8.1). Titration of TM
780 concentration ensures less than one cluster of red/green cells per brain hemisphere and
781 maximizes the probability of analyzing a single clone (step 2.2)[7,11]. Clones should be discarded if
782 neighboring clusters of cells occur within 500 μm of the clone of interest. Therefore, it is
783 important to examine several sections before and after the appearance of a clone to ensure that
784 there are no additional recombination events nearby. Due to weaker signal of the fluorophores,
785 it is necessary to perform immunohistochemistry for eGFP and tdT in embryonic clones (see
786 section 6). This is only recommended in adult clones if additional antigens will be colabeled.
787 When imaging clones, it is important to capture the entire width of the cortex where the clone is
788 located (i.e., from pial surface to the corpus callosum; see step 8.4) to not miss any cells. This also
789 facilitates image alignment during image processing (section 9). Section 8 of the protocol requires
790 an inverted confocal microscope but can be adapted depending on the microscope setup
791 available. Epifluorescence microscopy can be used, but confocal microscopy is recommended
792 because this leads to a decrease in light contamination from outside the focus plane. It is also

793 important that the laser intensity and gain is adjusted so that green, red, and yellow cells can be
794 unambiguously identified. Regardless of the setup, it is recommended to use an objective of at
795 least 20x to ensure full spatial separation of closely positioned cells. In addition to recording the
796 cortical depth of all cells (step 8.6), cortical regions where the clones are located must be
797 identified using a brain atlas such as the Allen Brain atlas or other stereotaxic coordinate maps.
798 A file naming paradigm should also be adopted to make sure clone images are easily identifiable.
799 The following information could be included in the file naming: unique image ID, date image was
800 taken, genotype of animal, age of induction, age of analysis, image number in relation to the rest
801 of the images from the same clone.

802

803 Introduction of a mutation distal to one MADM cassette distinctively allows the generation of
804 genetic mosaics[71] and permits the dissection of molecular regulators of lineage and cell type
805 diversity at the clonal level[7,11,46,62]. To generate a genetic mosaic with MADM, the MADM
806 cassettes must be meiotically linked to the same chromosome as the gene of interest (see **Figure
807 2** for breeding scheme). This limits current clonal analysis with MADM to genes located on Chr.
808 7[51], Chr. 11[46], Chr. 12[51], and Chr. 6 distal to the *Rosa26* locus[47]. Future studies will use MADM
809 cassettes targeted to any chromosome, permitting the mosaic analysis of virtually all genes of
810 the mouse genome at the clonal level.

811

812 Finally, MADM is not limited to the analysis of progenitor cells in the developing neocortex. The
813 study of many stem cell niches could benefit from the ability to resolve spatiotemporal
814 arrangements of clonally related cells. By applying MADM to other regions of the brain, disease
815 conditions (e.g., cancer), or in other tissues[47,50-59], studies revealed lineage relationships in clones
816 derived from diverse classes of progenitor and stem cells (see **Table 1** for current list of MADM
817 clonal studies). Another interesting future application of MADM is to combine it with additional
818 functional or subcellular reporters, which would increase the degree of information that can be
819 acquired from clones.

820

832

833 **DISCLOSURES:**
834 The authors have nothing to disclose.

835

836 **REFERENCES:**

837   1. Malatesta, P. et al. Neuronal or glial progeny: regional differences in radial glia fate. *Neuron.*
838   **37** (5), 751-764 (2003).
839   2. Miyata, T., Kawaguchi, A., Okano, H., Ogawa, M. Asymmetric inheritance of radial glial fibers
840   by cortical neurons. *Neuron.* **31** (5), 727-741 (2001).
841   3. Noctor, S. C., Flint, A. C., Weissman, T. A., Dammerman, R. S., Kriegstein, A. R. Neurons derived
842   from radial glial cells establish radial units in neocortex. *Nature.* **409** (6821), 714-720 (2001).
843   4. Taverna, E., Götz, M., Huttner, W. B. The Cell Biology of Neurogenesis: Toward an
844   Understanding of the Development and Evolution of the Neocortex. *Annual Review of Cell and*
845   *Developmental Biology.* **30** (1), 465-502 (2014).
846   5. Desikan, R. S., Barkovich, A. J. Malformations of cortical development. *Annals of Neurology.* **80**
847   (6), 797-810 (2016).
848   6. Gao, R., Penzes, P. Common mechanisms of excitatory and inhibitory imbalance in
849   schizophrenia and autism spectrum disorders. *Current Molecular Medicine.* **15** (2), 146-167
850   (2015).
851   7. Beattie, R. et al. Mosaic Analysis with Double Markers Reveals Distinct Sequential Functions of
852   Lgl1 in Neural Stem Cells. *Neuron.* **94** (3), 517-533.e3 (2017).
853   8. Hippenmeyer, S. Molecular pathways controlling the sequential steps of cortical projection
854   neuron migration. *Advances in Experimental Medicine and Biology.* **800**, 1-24 (2014).
855   9. Lodato, S., Arlotta, P. Generating neuronal diversity in the mammalian cerebral cortex. *Annual*
856   *Review of Cell and Developmental Biology.* **31**, 699-720 (2015).
857   10. Hansen, A. H., Duellberg, C., Mieck, C., Loose, M., Hippenmeyer, S. Cell Polarity in Cerebral
858   Cortex Development—Cellular Architecture Shaped by Biochemical Networks. *Frontiers in*
859   *Cellular Neuroscience.* **11**, 176 (2017).
860   11. Gao, P. et al. Deterministic progenitor behavior and unitary production of neurons in the
861   neocortex. *Cell.* **159** (4), 775-788 (2014).
862   12. Llorca, A. et al. A stochastic framework of neurogenesis underlies the assembly of neocortical
863   cytoarchitecture. *eLife.* **8**, e51381 (2019).
864   13. Ma, J., Shen, Z., Yu, Y.-C., Shi, S.-H. Neural lineage tracing in the mammalian brain. *Current*
865   *Opinion in Neurobiology.* **50**, 7-16 (2018).
866   14. Caviness, V., Takahashi, T., Nowakowski, R. Numbers, time and neocortical neuronogenesis:
867   a general developmental and evolutionary model. *Trends in Neurosciences.* **18** (9), 379-383
868   (1995).
869   15. Schmechel, D. E., Rakic, P. A Golgi study of radial glial cells in developing monkey
870   telencephalon: morphogenesis and transformation into astrocytes. *Anatomy and Embryology.*
871   **156** (2), 115-152 (1979).
872   16. Kessaris, N. et al. Competing waves of oligodendrocytes in the forebrain and postnatal
873   elimination of an embryonic lineage. *Nature Neuroscience.* **9** (2), 173-179 (2006).
874   17. Magavi, S., Friedmann, D., Banks, G., Stolfi, A., Lois, C. Coincident generation of pyramidal
875   neurons and protoplasmic astrocytes in neocortical columns. *The Journal of Neuroscience.* **32**
876   (14), 4762-4772 (2012).
877   18. Anthony, T. E., Klein, C., Fishell, G., Heintz, N. Radial glia serve as neuronal progenitors in all
878   regions of the central nervous system. *Neuron.* **41** (6), 881-890 (2004).
879   19. Voigt, T. Development of glial cells in the cerebral wall of ferrets: direct tracing of their
880   transformation from radial glia into astrocytes. *The Journal of Comparative Neurology.* **289** (1),

881   74-88 (1989).

882   20. Amberg, N., Laukoter, S., Hippenmeyer, S. Epigenetic cues modulating the generation of cell-
883   type diversity in the cerebral cortex. *Journal of Neurochemistry.* **149** (1), 12-26 (2019).

884   21. Beattie, R., Hippenmeyer, S. Mechanisms of Radial Glia Progenitor Cell Lineage Progression.
885   *FEBS letters.* **591** (24), 3993-4008 (2017).

886   22. Telley, L. et al. Temporal patterning of apical progenitors and their daughter neurons in the
887   developing neocortex. *Science.* **364** (6440), eaav2522 (2019).

888   23. Oberst, P. et al. Temporal plasticity of apical progenitors in the developing mouse neocortex.
889   *Nature.* **573** (7774), 370-374 (2019).

890   24. Telley, L. et al. Sequential transcriptional waves direct the differentiation of newborn neurons
891   in the mouse neocortex. *Science.* **351** (6280), 1443 (2016).

892   25. Deppe, U. et al. Cell lineages of the embryo of the nematode Caenorhabditis elegans.
893   *Proceedings of the National Academy of Sciences of the United States of America.* **75** (1), 376-380
894   (1978).

895   26. Woodworth, M. B., Girskis, K. M., Walsh, C. A. Building a lineage from single cells: genetic
896   techniques for cell lineage tracking. *Nature Reviews Genetics.* **18** (4), 230-244 (2017).

897   27. Masuyama, N., Mori, H., Yachie, N. DNA barcodes evolve for high-resolution cell lineage
898   tracing. *Current Opinion in Chemical Biology.* **52**, 63-71 (2019).

899   28. Legue, E., Joyner, A. L. Chapter Ten-Genetic Fate Mapping Using Site-Specific Recombinases.
900   *Methods in Enzymology.* **477**, 153-181 (2010).

901   29. Postiglione, M. P., Hippenmeyer, S. Monitoring neurogenesis in the cerebral cortex: an
902   update. *Future Neurology.* **9** (3), 323-340 (2014).

903   30. Espinosa-Medina, I., Garcia-Marques, J., Cepko, C., Lee, T. High-throughput dense
904   reconstruction of cell lineages. *Open Biology.* **9** (12), 190229 (2019).

905   31. Hwang, B. et al. Lineage tracing using a Cas9-deaminase barcoding system targeting
906   endogenous L1 elements. *Nature Communications.* **10** (1), 1234 (2019).

907   32. Kalhor, R. et al. Developmental barcoding of whole mouse via homing CRISPR. *Science.* **361**
908   (6405), eaat9804 (2018).

909   33. García-Moreno, F., Vasistha, N. A., Begbie, J., Molnár, Z. CLoNe is a new method to target
910   single progenitors and study their progeny in mouse and chick. *Development.* **141** (7), 1589-1598
911   (2014).

912   34. Loulier, K. et al. Multiplex Cell and Lineage Tracking with Combinatorial Labels. *Neuron.* **81**
913   (3), 505-520 (2014).

914   35. Livet, J. et al. Transgenic strategies for combinatorial expression of fluorescent proteins in the
915   nervous system. *Nature.* **450** (7166), 56-62 (2007).

916   36. Snippert, H. J. et al. Intestinal Crypt Homeostasis Results from Neutral Competition between
917   Symmetrically Dividing Lgr5 Stem Cells. *Cell.* **143** (1), 134-144 (2010).

918   37. Amitai-Lange, A. et al. A method for lineage tracing of corneal cells using multi-color
919   fluorescent reporter mice. *Journal of Visualized Experiments.* (106), e53370 (2015).

920   38. Vasistha, N. A. et al. Cortical and Clonal Contribution of Tbr2 Expressing Progenitors in the
921   Developing Mouse Brain. *Cerebral Cortex.* **25** (10), 3290-3302 (2015).

922   39. Chen, F., LoTurco, J. A method for stable transgenesis of radial glia lineage in rat neocortex
923   by piggyBac mediated transposition. *Journal of Neuroscience Methods.* **207** (2), 172-180 (2012).

924   40. Siddiqi, F. et al. Fate mapping by piggyBac transposase reveals that neocortical GLAST+

925   progenitors generate more astrocytes than Nestin+ progenitors in rat neocortex. *Cerebral Cortex.*
926   **24** (2), 508-520 (2014).

927   41. Yoshida, A. et al. Simultaneous expression of different transgenes in neurons and glia by
928   combining in utero electroporation with the Tol2 transposon-mediated gene transfer system.
929   *Genes to Cells.* **15** (5), 501-512 (2010).

930   42. García-Marqués, J., López-Mascaraque, L. Clonal Identity Determines Astrocyte Cortical
931   Heterogeneity. *Cerebral Cortex.* **23** (6), 1463-1472 (2013).

932   43. Clavreul, S. et al. Cortical astrocytes develop in a plastic manner at both clonal and cellular
933   levels. *Nature Communications.* **10** (1), 4884 (2019).

934   44. Kim, G. B. et al. Rapid Generation of Somatic Mouse Mosaics with Locus-Specific, Stably
935   Integrated Transgenic Elements. *Cell.* **179** (1), 251-267.e224 (2019).

936   45. Pilz, G.-A. et al. Live imaging of neurogenesis in the adult mouse hippocampus. *Science.* **359**
937   (6376), 658 (2018).

938   46. Hippenmeyer, S. et al. Genetic Mosaic Dissection of Lis1 and Ndel1 in Neuronal Migration.
939   *Neuron.* **68** (4), 695-709 (2010).

940   47. Zong, H., Espinosa, J. S., Su, H. H., Muzumdar, M. D., Luo, L. Mosaic analysis with double
941   markers in mice. *Cell.* **121** (3), 479-492 (2005).

942   48. Ramón y Cajal, S. *Histologie du système nerveux de l'homme et des vertébrés.* Maloine. Paris,
943   France (1911).

944   49. Cowan, W. M. The emergence of modern neuroanatomy and developmental neurobiology.
945   *Neuron.* **20** (3), 413-426 (1998).

946   50. Ali, S. R. et al. Existing cardiomyocytes generate cardiomyocytes at a low rate after birth in
947   mice. *Proceedings of the National Academy of Sciences of the United States of America.* **111** (24),
948   8850-8855 (2014).

949   51. Hippenmeyer, S., Johnson, R. L., Luo, L. Mosaic analysis with double markers reveals cell-type-
950   specific paternal growth dominance. *Cell Reports.* **3** (3), 960-967 (2013).

951   52. Riccio, P., Cebrian, C., Zong, H., Hippenmeyer, S., Costantini, F. Ret and Etv4 promote directed
952   movements of progenitor cells during renal branching morphogenesis. *PLoS Biology.* **14** (2),
953   e1002382 (2016).

954   53. Bonaguidi, M. A. et al. In vivo clonal analysis reveals self-renewing and multipotent adult
955   neural stem cell characteristics. *Cell.* **145** (7), 1142-1155 (2011).

956   54. Mayer, C. et al. Clonally Related Forebrain Interneurons Disperse Broadly across Both
957   Functional Areas and Structural Boundaries. *Neuron.* **87** (5), 989-998 (2015).

958   55. Muzumdar, M. D. et al. Clonal dynamics following p53 loss of heterozygosity in Kras-driven
959   cancers. *Nature Communications.* **7**, 12685 (2016).

960   56. Shi, W. et al. Ontogenetic establishment of order-specific nuclear organization in the
961   mammalian thalamus. *Nature Neuroscience.* **20**, 516 (2017).

962   57. Wong, S. Z. H. et al. In vivo clonal analysis reveals spatiotemporal regulation of thalamic
963   nucleogenesis. *PLoS Biology.* **16** (4), e2005211 (2018).

964   58. Xu, H.-T. et al. Distinct Lineage-Dependent Structural and Functional Organization of the
965   Hippocampus. *Cell.* **157** (7), 1552-1564 (2014).

966   59. Brennand, K., Huangfu, D., Melton, D. All β Cells Contribute Equally to Islet Growth and
967   Maintenance. *PLoS Biology.* **5** (7), e163 (2007).

968   60. Ortiz-Alvarez, G. et al. Adult neural stem cells and multiciliated ependymal cells share a

969   common lineage regulated by the geminin family members. *Neuron.* **102** (1), 159-172. e157
970   (2019).

971   61. Kaplan, E. S., Ramos-Laguna, K. A., Mihalas, A. B., Daza, R. A. M., Hevner, R. F. Neocortical
972   Sox9+ radial glia generate glutamatergic neurons for all layers, but lack discernible evidence of
973   early laminar fate restriction. *Neural Development.* **12** (1), 14 (2017).

974   62. Lv, X. et al. TBR2 coordinates neurogenesis expansion and precise microcircuit organization
975   via Protocadherin 19 in the mammalian cortex. *Nature Communications.* **10** (1), 3946 (2019).

976   63. Mihalas, A. B., Hevner, R. F. Clonal analysis reveals laminar fate multipotency and daughter
977   cell apoptosis of mouse cortical intermediate progenitors. *Development.* **145** (17), dev164335
978   (2018).

979   64. Picco, N. et al. A mathematical insight into cell labelling experiments for clonal analysis.
980   *Journal of Anatomy.* **235** (3), 687-696 (2019).

981   65. Johnson, C. A., Ghashghaei, H. T. Sp2 regulates late neurogenic but not early expansive
982   divisions of neural stem cells underlying population growth in the mouse cortex. *Development.*
983   dev.186056 (2020).

984   66. Espinosa, J. S., Luo, L. Timing Neurogenesis and Differentiation: Insights from Quantitative
985   Clonal Analyses of Cerebellar Granule Cells. *The Journal of Neuroscience.* **28** (10), 2301 (2008).

986   67. Schneider, C. A., Rasband, W. S., Eliceiri, K. W. NIH Image to ImageJ: 25 years of image
987   analysis. *Nature Methods.* **9** (7), 671-675 (2012).

988   68. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. *Nature
989   Methods.* **9** (7), 676-682 (2012).

990   69. Liu, Y. et al. Tamoxifen-independent recombination in the RIP-CreER mouse. *PLoS One.* **5** (10),
991   e13533 (2010).

992   70. Klingler, E., Jabaudon, D. Do progenitors play dice? *eLife.* **9**, e54042 (2020).

993   71. Hippenmeyer, S. Dissection of gene function at clonal level using mosaic analysis with double
994   markers. *Frontiers in Biology.* **8** (6), 557-568 (2013).

995   72. Rulands, S., Simons, B. D. Tracing cellular dynamics in tissue development, maintenance and
996   disease. *Current Opinion in Cell Biology.* **43**, 38-45 (2016).

997   73. Laukoter, S. et al. Imprinted Cdkn1c genomic locus cell-autonomously promotes cell survival
998   in cerebral cortex development. *Nature Communications.* **11** (1), 195 (2020).

999   74. Daigle, T. L. et al. A Suite of Transgenic Driver and Reporter Mouse Lines with Enhanced Brain-
1000   Cell-Type Targeting and Functionality. *Cell.* **174** (2), 465-480.e422 (2018).

1001   75. He, M. et al. Strategies and Tools for Combinatorial Targeting of GABAergic Neurons in Mouse
1002   Cerebral Cortex. *Neuron.* **91** (6), 1228-1243 (2016).

1003   76. Yamamoto, M. et al. A multifunctional reporter mouse line for Cre- and FLP-dependent
1004   lineage analysis. *Genesis.* **47** (2), 107-114 (2009).

1005   77. Plummer, N. W. et al. Expanding the power of recombinase-based labeling to uncover cellular
1006   diversity. *Development.* **142** (24), 4385 (2015).

1007   78. Imayoshi, I., Ohtsuka, T., Metzger, D., Chambon, P., Kageyama, R. Temporal regulation of Cre
1008   recombinase activity in neural stem cells. *Genesis.* **44** (5), 233-238 (2006).

1009   79. Sasaki, S. et al. Complete loss of Ndel1 results in neuronal migration defects and early
1010   embryonic lethality. *Molecular and Cellular Biology.* **25** (17), 7812-7827 (2005).

1011   80. Acampora, D. et al. Epilepsy and brain abnormalities in mice lacking the Otx1 gene. *Nature
1012   Genetics.* **14** (2), 218-222 (1996).

1013   81. Soeda, T. et al. Sox9-expressing precursors are the cellular origin of the cruciate ligament of
1014   the knee joint and the limb tendons. *Genesis.* **48** (11), 635-644 (2010).
1015   82. Klezovitch, O., Fernandez, T. E., Tapscott, S. J., Vasioukhin, V. Loss of cell polarity causes
1016   severe brain dysplasia in Lgl1 knockout mice. *Genes & Development.* **18** (5), 559-571 (2004).
1017   83. Pimeisl, I. M. et al. Generation and characterization of a tamoxifen-inducible EomesCreER
1018   mouse line. *Genesis.* **51** (10), 725-733 (2013).
1019   84. Nakagawa, N. et al. Memo1-Mediated Tiling of Radial Glial Cells Facilitates Cerebral Cortical
1020   Development. *Neuron.* **103** (5), 836-852.e835 (2019).
1021   85. Nowotschin, S. et al. The T-box transcription factor Eomesodermin is essential for AVE
1022   induction in the mouse embryo. *Genes & Development.* **27** (9), 997-1002 (2013).
1023   86. Balordi, F., Fishell, G. Mosaic removal of hedgehog signaling in the adult SVZ reveals that the
1024   residual wild-type stem cells have a limited capacity for self-renewal. *Journal of Neuroscience.* **27**
1025   (52), 14248-14259 (2007).
1026   87. Liang, H. et al. Neural development is dependent on the function of specificity protein 2 in
1027   cell cycle progression. *Development.* **140** (3), 552-561 (2013).
1028   88. Guo, C., Yang, W., Lobe, C. G. A Cre recombinase transgene with mosaic, widespread
1029   tamoxifen-inducible action. *Genesis.* **32** (1), 8-18 (2002).
1030   89. Ahn, S., Joyner, A. L. In vivo analysis of quiescent adult neural stem cells responding to Sonic
1031   hedgehog. *Nature.* **437** (7060), 894-897 (2005).
1032   90. Storm, R. et al. The bHLH transcription factor Olig3 marks the dorsal neuroepithelium of the
1033   hindbrain and is essential for the development of brainstem nuclei. *Development.* **136** (2), 295-
1034   305 (2009).
1035   91. Koundakjian, E. J., Appler, J. L., Goodrich, L. V. Auditory neurons make stereotyped wiring
1036   decisions before maturation of their targets. *Journal of Neuroscience.* **27** (51), 14078-14088
1037   (2007).
1038   92. Dor, Y., Brown, J., Martinez, O. I., Melton, D. A. Adult pancreatic β-cells are formed by self-
1039   duplication rather than stem-cell differentiation. *Nature.* **429** (6987), 41-46 (2004).
1040   93. Sohal, D. S. et al. Temporally regulated and tissue-specific gene manipulations in the adult
1041   and embryonic heart using a tamoxifen-inducible Cre protein. *Circulation Research.* **89** (1), 20-25
1042   (2001).
1043   94. Ventura, A. et al. Restoration of p53 function leads to tumour regression in vivo. *Nature.* **445**
1044   (7128), 661-665 (2007).
1045   95. Johnson, L. et al. Somatic activation of the K-ras oncogene causes early onset lung cancer in
1046   mice. *Nature.* **410** (6832), 1111-1116 (2001).
1047   96. Tasic, B. et al. Extensions of MADM (mosaic analysis with double markers) in mice. *PLoS One.*
1048   **7** (3), e33332 (2012).
1049   97. Yu, J., Carroll, T. J., McMahon, A. P. Sonic hedgehog regulates proliferation and differentiation
1050   of mesenchymal cells in the mouse metanephric kidney. *Development.* **129** (22), 5301-5312
1051   (2002).
1052   98. Zhao, H. et al. Role of fibroblast growth factor receptors 1 and 2 in the ureteric bud.
1053   *Developmental Biology.* **276** (2), 403-415 (2004).
1054   99. Schuchardt, A., D'Agati, V., Larsson-Blomberg, L., Costantini, F., Pachnis, V. Defects in the
1055   kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. *Nature.* **367**
1056   (6461), 380-383 (1994).

1057   100. Livet, J. et al. ETS gene Pea3 controls the central position and terminal arborization of
1058   specific motor neuron pools. *Neuron.* **35** (5), 877-892 (2002).

1059   101. Volz, K. S. et al. Pericytes are progenitors for coronary artery smooth muscle. *eLife.* **4**,
1060   e10036 (2015).

1061   102. Cai, C.-L. et al. A myocardial lineage derives from Tbx18 epicardial cells. *Nature.* **454** (7200),
1062   104-108 (2008).

1063   103. Lasrado, R. et al. Lineage-dependent spatial and functional organization of the mammalian
1064   enteric nervous system. *Science.* **356** (6339), 722-726 (2017).

1065   104. Matsuoka, T. et al. Neural crest origins of the neck and shoulder. *Nature.* **436** (7049), 347-
1066   355 (2005).

1067

Fig. 1

Figure 2

Fig. 2

Time Course Paradigms for MADM-based Lineage Analysis in Cerebral Cortex

E0/Plug E10 E12 E14 E16 E18 P0 P4 P7 P14 P21 P28 Time

Longitudinal sampling

Progressive interval sampling

Fig. 3

Fig. 4

Fig. 5

Figure 6
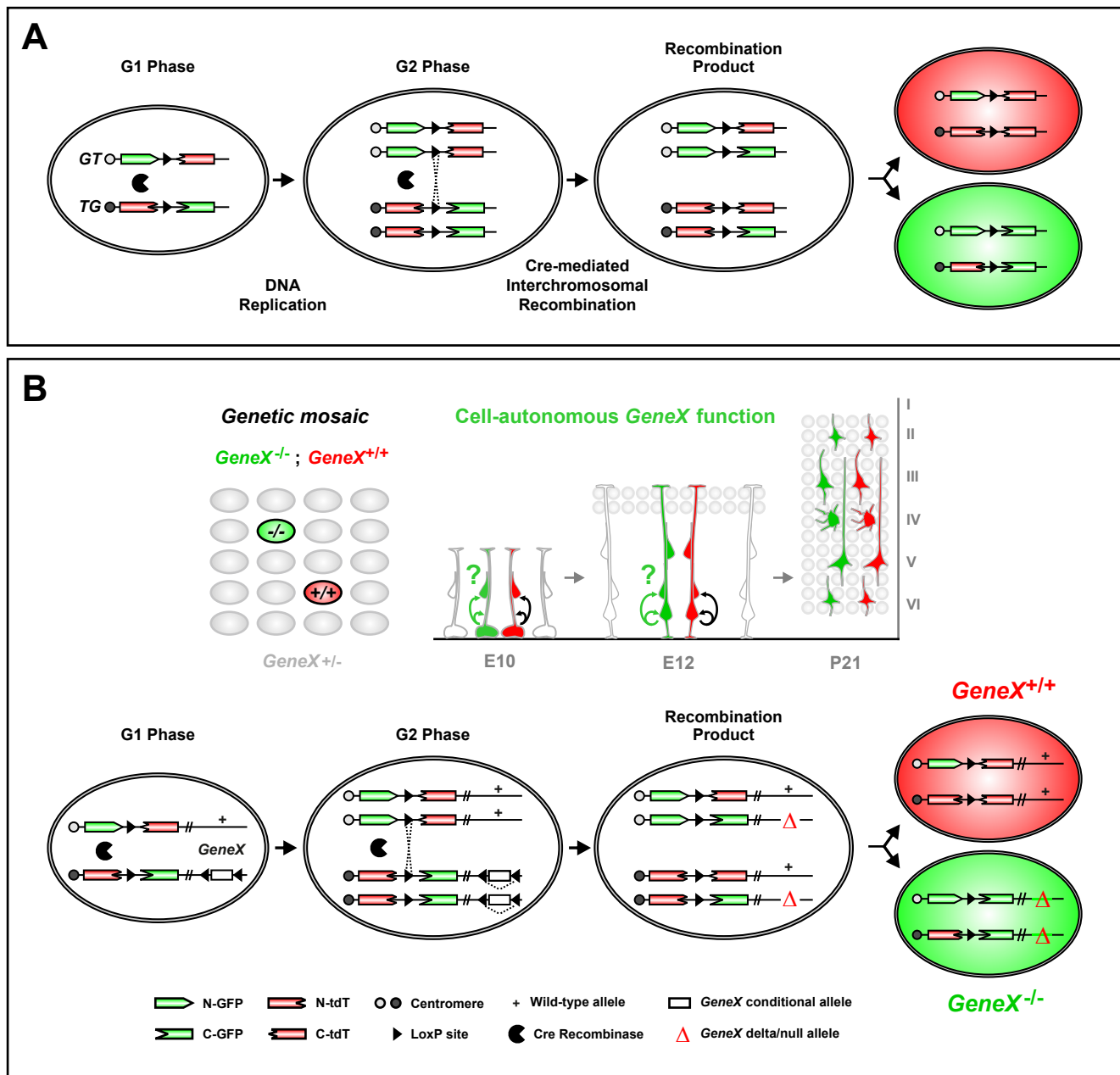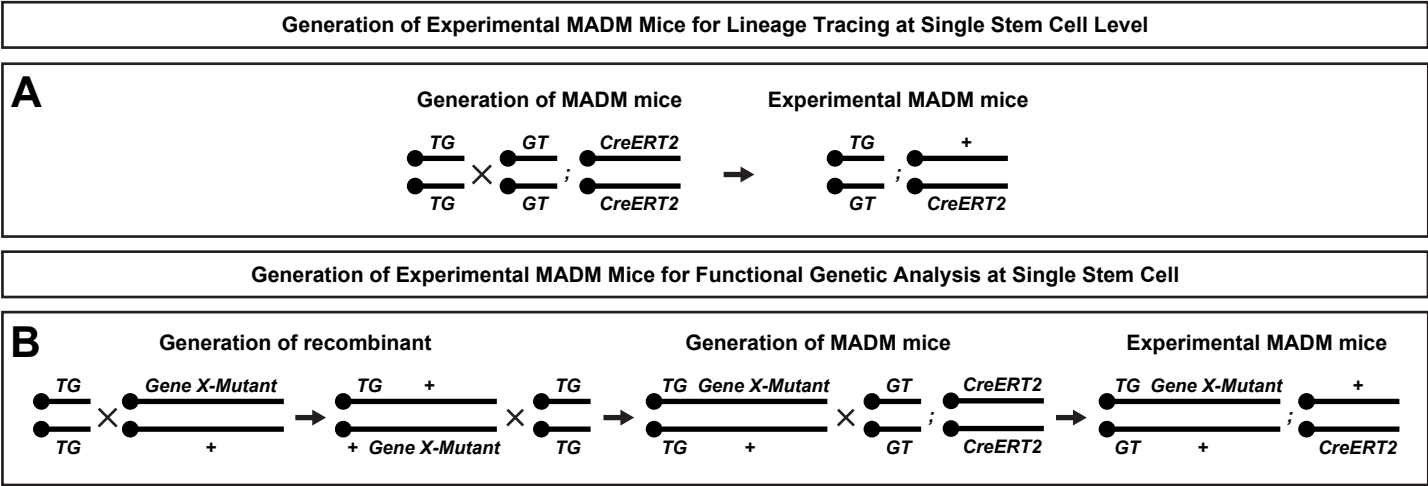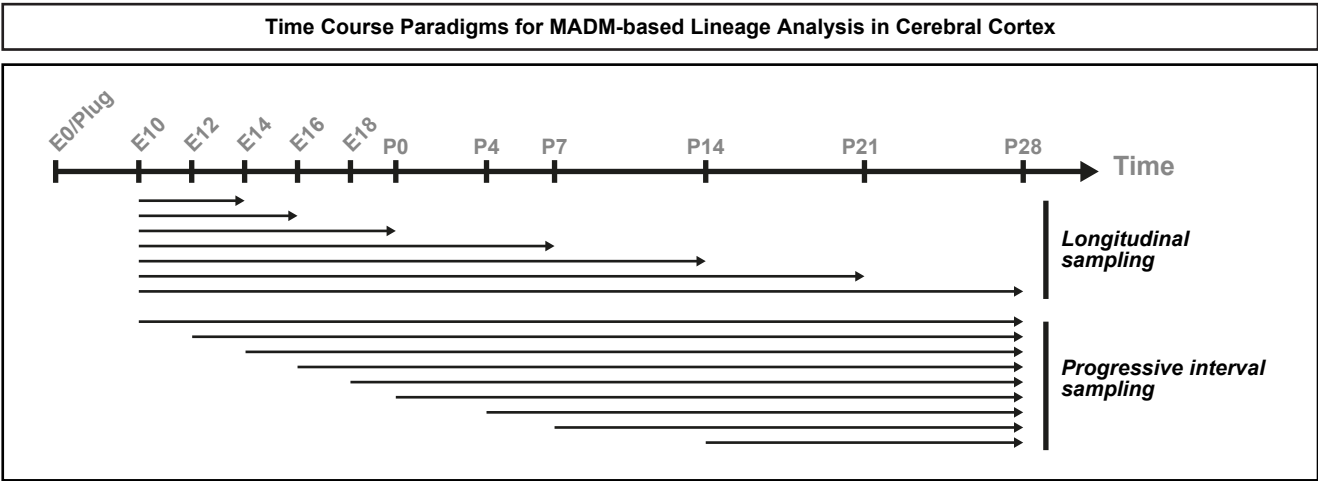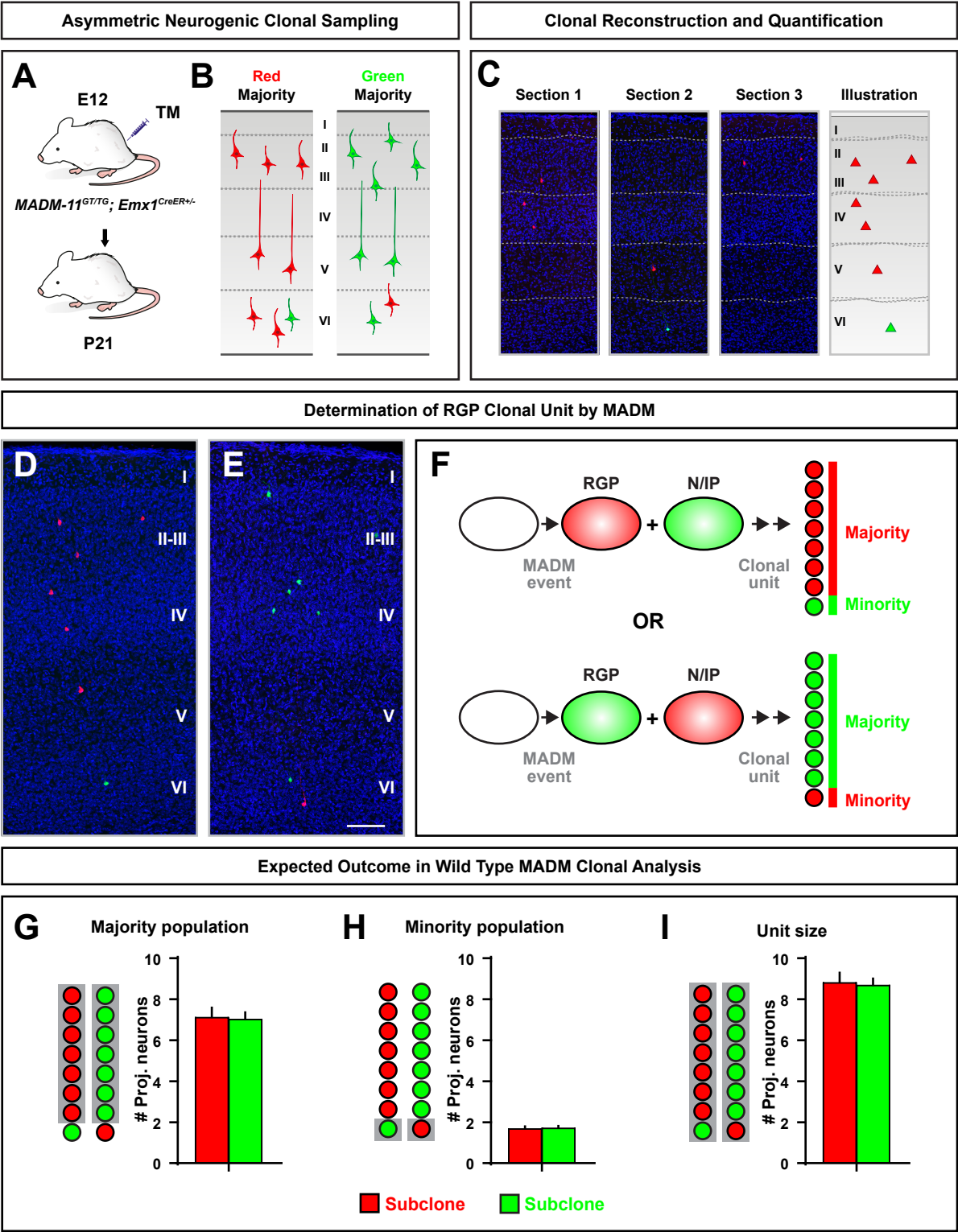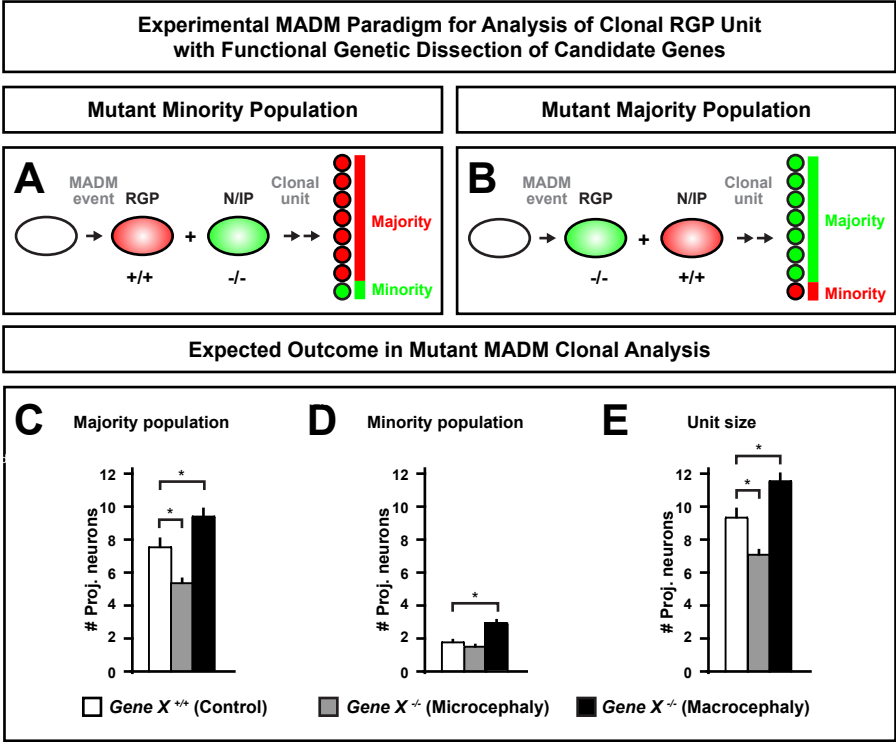
Fig. 6

MADM Clonal Analysis Study	Tissue Study Was Performed In	MADM Cassette	CreER[T2] Driver	Mutant Allele	Tamoxifen Induction Paradigm
Hippenmeyer et al., 2010[46]	RGPs in the cortex	MADM-11 (JAX Stock 013749 and 013751)	Nestin-CreER[T2] (Line 5 from Imayoshi et al., 2006)[78]	Ndel1 (Sasaki et al., 2005)[79]	Single IP injection 1–3 mg/mouse at E8 or E10
Gao et al., 2014[11]	RGPs in the cortex	MADM-11 (JAX Stock 013749 and 013751)	Emx1-CreER[T2] (Kessaris et al., 2006)[16] and Nestin-CreER[T2] (Line 5 from Imayoshi et al., 2006)[78]	Otx1 (Acampora et al., 1996)[80]	Single IP injection at E10, E11, E12, or E13 or orally gavaged with at E15 at a dose of 25–50 mg/kg of body weight.
Kaplan et al., 2017[61]	RGPs in the cortex	MADM-11 (JAX Stock 013749 and 013751)	Sox9-CreER[T2] (Soeda et al., 2010)[81]	-	Single IP injection 100 mg/kg at E11.5
Beattie et al., 2017[7]	RGPs in the cortex	MADM-11 (JAX Stock 013749 and 013751)	Emx1-CreER[T2] (Kessaris et al., 2006)[16]	Lgl1 (Klezovitch et al., 2004)[82]	Single IP injection 2–3 mg/mouse at E11 or E12
Mihalas and Hevner, 2018[63]	Intermediate progenitors in the cortex	MADM-11 (JAX Stock 013749 and 013751)	Eomes-CreER[T2] (Tbr2) (Pimeisl et al., 2013)[83]	-	Single IP injection 30–75 mg/kg at E11.5, E12.5 or E13.5
Llorca et al., 2019[12]	RGPs in the cortex	MADM-11 (JAX Stock 013749 and 013751)	Emx1-CreER[T2] (Kessaris et al., 2006)[16]	-	Single IP injection 2–3 mg/mouse at E12
Picco et al., 2019[64]	RGPs in the cortex	MADM-11 (JAX Stock 013749 and 013751)	Emx1-CreER[T2] (Kessaris et al., 2006)[16]	-	Single IP injection 1–3 mg/mouse at E10, E11, E12, E13 or E14
Nakagawa et al., 2019*[84]	RGPs in the cortex	MADM-11 (JAX Stock 013749 and 013751)	Cre plasmid in utero electroporation	Memo1 (Nakagawa et al., 2019)[84]	Cre activity induced through IUE in MADM pregnant dams at E14 and analysed at E16.
Lv et al., 2019[62]	RGPs in the cortex	MADM-11 (JAX Stock 013749 and 013751)	Emx1-CreER[T2] (Kessaris et al., 2006)[16]	Tbr2 (Nowotschin, S. et al. 2013)[85]	Single IP injection 25–50 mg/kg at E10, E11, E12, or E13
Johnson and Ghashghaei, 2020[65]	RGPs in the cortex	MADM-11 (JAX Stock 013749 and 013751)	Nestin-CreER[T2] (Balordi and Fishell, 2007)[86]	Sp2 (Liang et al. 2013)[87]	Single IP injection 50 mg/kg at E11.5
Zong et al., 2005[47]	Cerebellar granule cell progenitors	MADM-6-ROSA (Zong et al., 2005)[47] (JAX Stock 006075 and 006080)	β-actin-CreER[T2] (Guo et al., 2002)[88]	-	Single IP injection 8–10 mg at E13.5 or E17.5
Espinosa et al., 2008[66]	Cerebellar granule cell progenitors	MADM-6-ROSA (Zong et al., 2005)[47] (JAX Stock 006075 and 006080)	β-actin-CreER[T2] (Guo et al., 2002)[88]	-	8 mg tamoxifen injections at E17.5
Bonaguidi et al., 2011[53]	Stem cells in postnatal dentate gyrus	MADM-6-ROSA (Zong et al., 2005)[47] (JAX Stock 006075 and 006080)	Nestin-CreER[T2] (Balordi and Fishell, 2007)[86]	-	Single IP injection 186–373 mg/kg in 8- to 10-week old mice
Xu et al., 2014[58]	RGPs in the hippocampus	MADM-11 (JAX Stock 013749 and 013751)	Emx1-CreER[T2] (Kessaris et al., 2006)[16]	-	Single IP injection at E10, E11, E12, or E13 at a dose of 25–50 mg/kg of body weight.
Mayer et al., 2015[54]	Interneuron progenitors in MGE	MADM-11 (JAX Stock 013749 and 013751)	Nestin-CreER[T2] (Line 5 from Imayoshi et al., 2006)[78]	-	Single IP injection 2–3 mg/mouse at E10
Shi et al., 2017[56]	Thalamic progenitors	MADM-11 (JAX Stock 013749 and 013751)	Nestin-CreER[T2] (Line 5 from Imayoshi et al., 2006)[78]	-	Single IP injection at E9, E10, E11, and E12 at a dose of 5–25 mg/kg body weight.
Wong et al., 2018[57]	Thalamic progenitors	MADM-11 (JAX Stock 013749 and 013751)	Gli1-CreER[T2] (Ahn et al., 2005)[89], Olig3-CreER[T2] (Storm et al., 2009)[90], and Neurog1-CreER[T2] (Koundakjian et al., 2007)[91]		A single dose of tamoxifen (132 mg/kg for Gli1-CreER[T2], 24 mg/kg or 34 mg/kg for Olig3-CreER[T2] and Neurog1-CreER[T2]). IP injection for Gli1-CreER[T2] and oral gavage for Olig3-CreER[T2] and Neurog1-CreER[T2] at various time points: E9.5 for Gli1-CreER[T2], E10.5 or E11.5 for Olig3-CreER[T2] and E11.5 to E14.5 for Neurog1-CreER[T2].
Ortiz-Alvarez et al. 2019*[60]	Ventricular-subventricular zone progenitors	MADM-11 (JAX Stock 013749 and 013751)	Cre plasmid in utero electroporation	-	Cre activity induced through IUE in MADM pregnant dams at E13.5 or E14.5 and analyzed the V-SVZ at P15–P20. This approach allowed a clonal study of green-red clones because the efficiency of recombination leading to green-red clones was low in these mice (mean number of clones per animal, 5).
Brennand et al., 2007[59]	β cells of the pancreas	MADM-6-ROSA (Zong et al., 2005)[47] (JAX Stock 006075 and 006080)	RIP-CreER[T2] (Dor et al., 2004)[92]	-	3 consecutive days of IP injections 6 or 8 mg/day in 4–8 week old mice.
Hippenmeyer et al., 2013[51]	Progenitors in liver	MADM-7 (JAX Stock 021457 and 021458)	β-actin-CreER[T2] (Guo et al., 2002)[88]	-	Single IP injection at E12
Ali et al., 2014[50]	α-myosin heavy chain expressing cardiomyocytes	MADM-11 (JAX Stock 013749 and 013751)	β-actin-CreER[T2] (Guo et al., 2002)[88] and Myh6-CreER[T2] (Sohal et al., 2001)[93]		Single IP injection 0.5 mg/pup at P4
Muzumdar et al., 2016[55]	Pancreatic tumour formation	MADM-11 (JAX Stock 013749 and 013751)	Rosa26-CreER[T2] (Ventura et al., 2007)[94]	Trp53 (Jax Stock Stock 008462) KrasLA2 (Johnson et al. 2001)[95]	Single IP injection 9 mg/40 g (3 doses) in 5–10 week old mice
Riccio et al., 2016*[52]	Primary ureteric bud progenitors in kidney development	MADM-6-ROSA (Tasic et al., 2012)[96], MADM-11 (JAX Stock 013749 and 013751)	Hoxb7-Cre (Yu et al., 2002)[97], Hoxb7-CreGFP (Zhao et al., 2004)[98]	Ret (Schuchardt et al., 1994)[99], Etv4 (Livet et al., 2002)[100]	-
Volz et al., 2015*[101]	Pericytes in the developing heart	MADM-11 (JAX Stock 013749 and 013751)	Tbx18-Cre (Cai et al., 2008)[102]	-	-
Lasrado et al., 2017*[103]	Vagal neural crest cells of the enteric nervous system.	MADM-6-ROSA (JAX Stock 006075 and 006080)	Sox10-Cre (Matsuoka et al., 2005)[104]	Ret (Schuchardt et al., 1994)[99]	-

*Used non-inducible cre

Table of Materials

Material/Reagent	Company	Order number	Comments
1 mL tuberculin syringe (Omnifix Luer Lock)	Braun	9204512N	
1,4-diazabicyclooctane (DABCO)	Roth	0718.2	
10 mL Syringe (Omnifix Luer Lock)	Braun	8508429N	
15 mL conical centrifuge	Sarstedt	65.554.502	
24 multi-well dishes	Roth/Greiner Bio-one	CE56.1	
27- gauge x 3/4 needle (Sterican)	Braun	16010256E	
Corn oil	Sigma	C8267-500ML	
Coverslips (24 x 60 mm #1)	Thermo Fisher Scientific (Menzel)	15747592	
Cryostat Cryostar NX70	Thermo Fisher Scientific	957000H	
Dako Pen (Wax marker)	Agilent	S200230-2	
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	Invitrogen	D1306	
Disposable microtome blade (MX35 Ultra)	Thermo Fisher Scientific	705830	
Fine Forceps (Dumont #5)	Fine Science Tools (FST)	11254-20	
Glass anti-roll plate	Histocom	M 449980	
Glycerol	Sigma	G5516	
LSM 800 Confocal	Zeiss		
Mounting medium			25 mg/mL DAPCO, 6 g Glycerol, 2.4 g Mowiol 4-88, 6 mL dH$_2$O, 12 mL 0.2 M Tris-HCl (pH 8.5)
Mowiol 4-88	Roth	0713.2	
Normal donkey serum	Innovative Research	IGDNSER100ML	
Paraformaldehyde	Sigma	441244-1KG	
Peristaltic pump 323E/D 400RPM	Watson-Marlow	036.3124.00A	
Sucrose	Sigma	S8501-5KG	
Superfrost plus glass slides	Thermo Fisher Scientific	J1800AMNT	
Tamoxifen	Sigma	T5648	
Tissue Embedding mold T-12 (22mm square)	Polysciences Inc.	18986-1	
Tissue-Tek O.C.T	Sakura	4583	
Triton X-100	Sigma	T8787-250ML	
Trizma hydrochloride	Sigma	93363	
Tween -20	Sigma	P9416-100ML	
Software and Plugins:			
Name	**Version**	**Source Link**	
Fiji	1.52p	Fiji	
MultiStackReg	1.45	Download link	
TurboReg		EPFL Bioimaging	
Zen Blue	2.6	Zeiss	
Experimental Models:			
Organisms/Strains	**Company**	**Order number**	
Mouse: *Emx1-CreER*	The Jackson Laboratory	JAX:027784	
Mouse: *MADM-11-GT*	The Jackson Laboratory	JAX:013749	
Mouse: *MADM-11-TG*	The Jackson Laboratory	JAX:013751	
Primary antibodies:			
Name/Dilution	**Company**	**Order number**	
Chicken anti-GFP 1:500	Aves Labs	GFP-1020	
Goat anti-tdTomato 1:500	Sicgen Antibodies	AB8181-200	
Rabbit anti-RFP 1:500	MBL	PM005	
Secondary antibodies:			
Name/Dilution	**Company**	**Order number**	
Donkey Anti-Chicken Alexa Fluor 488 1:500	Jackson Immuno Research	715-475-150	
Donkey Anti-Goat Cy3 1:500	Jackson Immuno Research	705-165-147	
Donkey Anti-Rabbit Cy3 1:500	Jackson Immuno Research	711-165-152	

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Line 367: Please include this product in the Table of Materials
The mounting medium consists of 25 mg/mL DAPCO, 6 g Glycerol, 2.4 g Mowiol 4-88, 6 mL dH2O, 12 mL 0.2M Tris-HCl (pH 8.5). The text and table of materials has been updated to reflect this.

Line 424: I rephrased this sentence. Please review for accuracy.
We approve of this rephrasing.

Line 473: Correct?
The step order is correct, however "repeat" has been rephrased to "Perform" to decrease confusion.

Line 539: Please remove the link from the manuscript text and include it in the Table of Materials.
We have moved this url to the table of materials.

Line 544: Please remove the link from the manuscript text and include it in the Table of Materials.
We have moved this url to the table of materials.

Line 551: How?
We have updated the text to elaborate on how this step is performed. The next text reads, "Split channels into DAPI (blue), GFP (green), and tdT (red) within "Color" option under "Image" tab."

Line 579: Please reference each panel of Figures 4-6 in this section.
We have included reference to each panel of Figures 4-6 in the Representative Results section.

Line 629: E11?
The editor is correct, and the "E" has now been added to fix this.

Line 680: Figure legends should be placed directly after the results section according to JoVE guidelines. Please update reference numbers accordingly.
We thank the editor for pointing this out and have updated the reference numbers accordingly.

Line 683: Please rephrase to avoid overlap with previously published text.
We have rephrased the text to avoid overlap with previously published text.

Line 700: Please update reference numbers here.
We have updated these references.

Line 724: Please update the reference number here.
We have updated these references.

Line 743: Please rephrase to avoid overlap with previously published text.
We have rephrased the text to avoid overlap with previously published text.

Line 747: Please update reference numbers here.
We have updated these references.

Line 763: Please rephrase to avoid overlap with previously published text.

We have rephrased the text to avoid overlap with previously published text.

Line 802: Please update reference numbers here.
We have updated these references.

Line 863: Please spell out journal names.
We have updated and ensured that the names of all journals are spelt out.

4. As we can only film 2.75 pages of the protocol, please review and shorten the highlighted portion to 2.75 pages. Note that the highlighted content should contain essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please note that the editor has made some minor changes (e.g., combining some shorter steps) to condense the highlighted content. Please do not highlight any steps describing anesthetization and euthanasia.
We have highlighted only the necessary steps, making for a script that is 96 lines (2.25 pages) long.

References

1   Hippenmeyer, S. *et al.* Genetic Mosaic Dissection of Lis1 and Ndel1 in Neuronal Migration. *Neuron.* **68** (4), 695-709, (2010).
2   Rulands, S. & Simons, B. D. Tracing cellular dynamics in tissue development, maintenance and disease. *Curr Opin Cell Biol.* **43** 38-45, (2016).

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