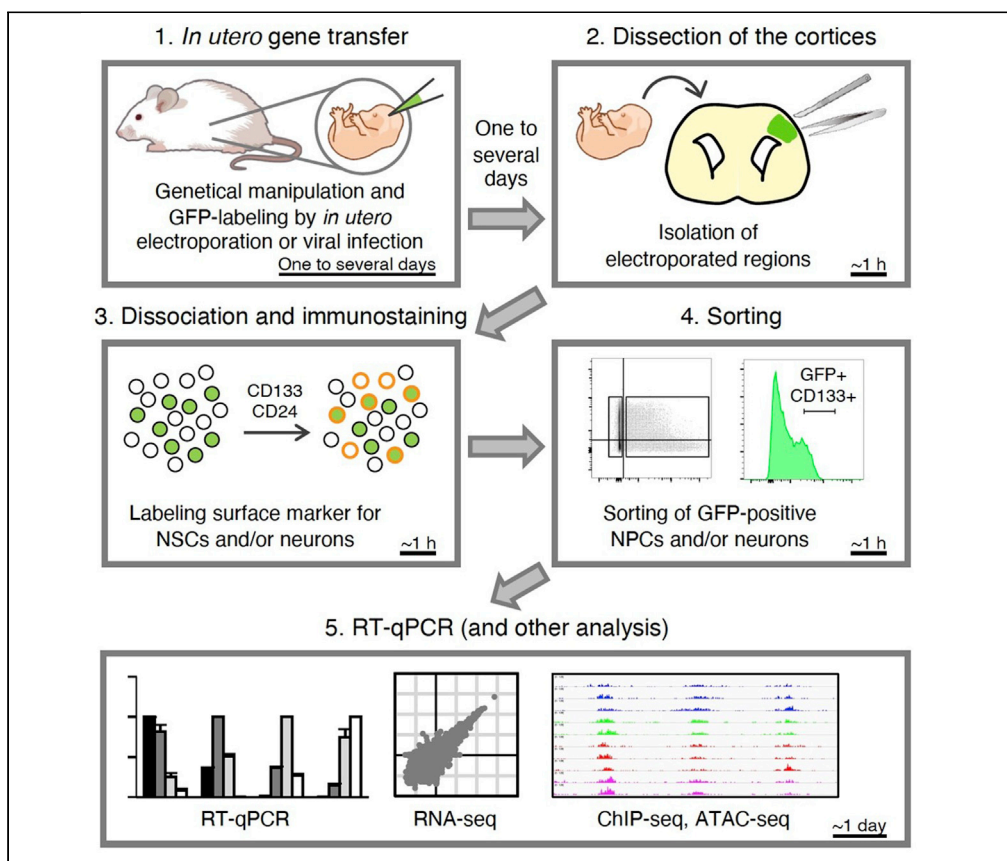


Protocol

Isolation of genetically manipulated neural progenitors and immature neurons from embryonic mouse neocortex by FACS



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Highlights

Direct isolation of neural progenitors and neurons from the mouse embryonic neocortex

Purification of neural cells at various stages of differentiation using wild-type mice

Protocol enables biochemical analyses of neural cells genetically manipulated *in vivo*

Applicable for transcriptomic and epigenomic analyses

The embryonic mammalian neocortex includes neural progenitors and neurons at various stages of differentiation. The regulatory mechanisms underlying multiple aspects of neocortical development—including cell division, neuronal fate commitment, neuronal migration, and neuronal differentiation—have been explored using *in utero* electroporation and virus infection. Here, we describe a protocol for investigation of the effects of genetic manipulation on neural development through direct isolation of neural progenitors and neurons from the mouse embryonic neocortex by fluorescence-activated cell sorting.

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Protocol

Isolation of genetically manipulated neural progenitors and immature neurons from embryonic mouse neocortex by FACS

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SUMMARY

The embryonic mammalian neocortex includes neural progenitors and neurons at various stages of differentiation. The regulatory mechanisms underlying multiple aspects of neocortical development—including cell division, neuronal fate commitment, neuronal migration, and neuronal differentiation—have been explored using in utero electroporation and virus infection. Here, we describe a protocol for investigation of the effects of genetic manipulation on neural development through direct isolation of neural progenitors and neurons from the mouse embryonic neocortex by fluorescence-activated cell sorting. For complete details on the use and execution of this protocol, please refer to Tsuboi et al. (2018) and Sakai et al. (2019).

BEFORE YOU BEGIN

In utero gene transfer

⌚ Timing: One to several days

The protocol is typically applied to mouse embryos that have been genetically manipulated by in utero electroporation or virus infection with a plasmid or viral vector encoding a protein or short hairpin RNA (shRNA) of interest as well as a fluorescent protein such as green fluorescent protein (GFP) (Gaiano et al., 1999; Kuwayama et al., 2020; Matsui et al., 2011; Tabata and Nakajima, 2001). It can also be applied to wild-type embryos as well as to transgenic embryos such as those that specifically express a Venus fluorescent protein in neural progenitors under the control of the rat *Nestin* enhancer (*Nestin-d4Venus* embryos) (Sunabori et al., 2008). For wild-type embryos, cell isolation is performed with antibodies to surface antigens of neural progenitors or of differentiated neurons, such as CD133 (a.k.a. prominin) and CD24, respectively (Pruszek et al., 2009; Uchida et al., 2000).

Note: We here describe the method to isolate neural cells from the mouse telencephalon at embryonic day (E) 8 or E9 or from the neocortex at E10 to 20 days postcoitus (dpc).

Note: For this protocol, we mainly use the ICR mouse strain, stage progression for which is slightly earlier compared with that for the C57BL/6 strain. For example, E12 of ICR roughly corresponds to E13 of C57BL/6.



Note: As described in the Limitations section, it is difficult to isolate neurons after they have elaborated neurites, given that enzymatic digestion of the elaborated neurites may impair the viability of or kill the cells.

Setup of FACS machine

⌚ Timing: ~1 h

1. Set up the FACSria instrument before dissection of embryos.
 - a. Turn on the FACSria, desktop computer, and lasers.
 - b. Perform prime after tank refill and fluidics startup.
 - c. Remove closed-loop nozzle and set the 100- μ m nozzle.
 - d. Turn on the stream and perform cytometer setup and tracking (CST) quality control.
 - e. Set Accudrop delay and check the sorting line by Test Sort.

Note: Nozzle size should be $\geq 100 \mu\text{m}$. The 85- μm nozzle may inflict more damage on neural cells.

Note: The neutral density (ND) filter should be >1.5 .

Preparation of dissociation solutions

⌚ Timing: 30 min

2. Thaw Neuron Dissociation Solutions (refer to [Key Resources Table](#)) in the indicated volume at step 5.

Note: We use thawed solutions that have been stored at 4°C for up to 1 month. For longer storage, we divide the solutions into smaller batches and refreeze them at -80°C .

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-CD133-PE (1:400)	BioLegend	Cat# 141204; RRID: AB_10722606
Anti-CD24-PE (1:400)	BioLegend	Cat# 138504; RRID: AB_10578416
Anti-CD133-APC (1:400)	BioLegend	Cat# 141208; RRID: AB_10896756
Chemicals, peptides, and recombinant proteins		
DMEM/F12	Sigma	Cat# D8062
Penicillin-streptomycin	Gibco	Cat# 15140-122
Percoll	Sigma	Cat# P1644-
BSA	FUJIFILM Wako Chemicals	Cat# 013-15143
NaCl	FUJIFILM Wako Chemicals	Cat# 191-01665
KCl	FUJIFILM Wako Chemicals	Cat# 163-03545
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	FUJIFILM Wako Chemicals	Cat# 196-02835
KH_2PO_4	FUJIFILM Wako Chemicals	Cat# 169-04245
NaOH	FUJIFILM Wako Chemicals	Cat# 194-18865
Critical commercial assays		
RNAiso plus	Takara	Cat# 9109
ReverTra Ace qPCR RT Master Mix with gDNA Remover	Toyobo	Cat# FSQ-301

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
THUNDERBIRD SYBR qPCR Mix	Toyobo	Cat# QPS-201T
Neuron Dissociation Solutions	FUJIFILM Wako Chemicals	Cat# 291-78001
Experimental models: organisms/strains		
Mouse: Jcl:ICR	CLEA Japan, Inc.	N/A
Mouse: Jcl:ICR	Japan SLC, Inc.	N/A
Mouse: Nestin-d4Venus	RIKEN BRC	RBRC04058
Oligonucleotides		
RT-qPCR primers, see Table 1	Eurofins Genomics	N/A
Software and algorithms		
FACS DIVA	BD	https://www.bdbiosciences.com/ja-jp/instruments/research-instruments/research-software/flow-cytometry-acquisition/facsdiva-software
FlowJo	BD	https://www.flowjo.com/solutions/flowjo
LightCycler 480 Software, version 1.5.1	Roche	https://lifescience.roche.com/global_en/products/lightcyler14301-480-software-version-15.html
Other		
5 mL Round Bottom Polystyrene Test Tube with Cell Strainer Snap Cap	Falcon	Cat# 352235
FACSria	BD	https://www.bdbiosciences.com/en-us/instruments/research-instruments/research-cell-sorters/facsria-iii
LightCycler 480	Roche	https://diagnostics.roche.com/global/en/products/systems/lightcyler-480-system.html#productInfo
NanoDrop	Thermo Fisher	https://www.thermofisher.com/jp/en/home/industrial/spectroscopy-elemental-isotope-analysis/molecular-spectroscopy/ultraviolet-visible-visible-spectrophotometry-uv-vis-vis/uv-vis-vis-instruments/nanodrop-microvolume-spectrophotometers/nanodrop-products-guide.html

MATERIALS AND EQUIPMENT

FACS

This protocol is based on the use of a FACSria instrument (BD) for isolation of neural cells. Potential alternative machines are MoFlo (Beckman Coulter) and Cell Sorter SH800S (Sony), which we have confirmed to be suitable for sorting of neural cells.

qPCR system

This protocol describes the quantitative polymerase chain reaction (qPCR) procedure as performed in a 5- μ l reaction volume with a LightCycler 480 system (Roche) and THUNDERBIRD SYBR qPCR Mix. Other qPCR machines, such as LC96 (Roche), and other reagents based on SYBR, such as a KAPA SYBR Fast qPCR Kit (KAPA Biosystems), LightCycler 480 SYBR Green I Master (Roche), and a QuantiNova SYBR Green PCR Kit (Qiagen), can also be used.

Table 1. Primer sequences for RT-qPCR

Gene	Forward primer	Reverse primer
<i>Gapdh</i>	ATGAATACGGCTACAGCAACAGG	CTCTTGCTCAGTGTCTTGCTG
<i>Nestin</i>	TGAAGCACTGGGAAGAGTAG	TAACTCATCTGCCTCACTGTC
<i>Sox2</i>	CATGAGAGCAAGTACTGGCAAG	CCAACGATATCAACCTGCATGG
<i>Pax6</i>	CGGAGGGAGTAAGCCAAGAG	TCTGTCTCGGATTTCCCAAG
<i>Eomes</i>	CATGGACATCCAGAATGAGC	CAGGAGGAACAACTCTTCTTTAAC
<i>Neurod1</i>	TACGACATGAACGGCTGCTA	TCTCCACCTTGCTCACTTT
<i>Tubb3</i>	ACACAGACGAGACCTACT	GCAGACACAAGGTGGTT
<i>Satb2</i>	GTCTCTCTGCCTCTAGC	GCGCCGTCCACCTTAATA
<i>Tbr1</i>	CCAAGGACCTGTCCGACTC	GCTCGTAAATCCCCGAGTC

10× phosphate-buffered saline (PBS)

Reagent	Final concentration	Amount
NaCl	1.37 M	320 g
KCl	26.8 mM	8 g
Na ₂ HPO ₄ ·12H ₂ O	101 mM	145.2 g
KH ₂ PO ₄	17.6 mM	9.6 g
ddH ₂ O		Up to 4 l
Total		4 l

Store at 18°C–24°C, and do not store more than 1 year.

Note: Adjust the pH of the solution with NaOH to 7.3.

0.2% BSA/PBS

Reagent	Final concentration	Amount
Bovine serum albumin (BSA)	0.2% (w/v)	0.1 g
10× PBS	1×	5 mL
ddH ₂ O		Up to 50 mL
Total		50 mL

Store at 4°C, and do not store more than 1 month.

DMEM/F12 containing 1% PS

Reagent	Final concentration	Amount
Penicillin-streptomycin (PS)	1% (v/v)	5 mL
DMEM/F12		Up to 500 mL
Total		500 mL

Store at 4°C, and do not store more than 1 month.

Alternatives: This protocol uses Dulbecco’s modified Eagle’s medium (DMEM)–nutrient mixture F12 containing 1% PS for dissection of the embryonic brain. Other appropriate solutions such as PBS and artificial cerebrospinal fluid can also be used.

STEP-BY-STEP METHOD DETAILS

Dissociation of embryonic mouse neocortex

⌚ Timing: 1–2 h

1. Add DMEM/F12 containing 1% PS to a glass dish cooled with ice or an ice pack.
2. Dissect the neocortex from embryos placed in the glass dish and isolate the region of interest with the use of a stereomicroscope.

Note: As a negative control, we isolate the neocortex of nonmanipulated embryos or nonmanipulated brain regions, such as the ventral area.

Note: When dissecting a neocortex subjected to electroporation with a fluorescent protein expression plasmid, it is possible to isolate only the electroporated region visible under a fluorescence stereomicroscope for efficient and fast cell sorting. However, for comparison of electroporated cells between neocortices transfected with test or control plasmids, the same neocortical region should be dissected to ensure the same CD133 distribution of the

fluorescent protein–negative cell population. This is important for use of the fluorescent protein–negative population as an appropriate internal control (Figure 1C).

Note: For the dissection of cortical tissues from mouse embryos, Viesselmann et al. (2011) provided useful a visual protocol (Viesselmann et al., 2011).

3. Transfer the neocortex into a 1.5-mL tube.
4. Leave the tube briefly to let the neocortex sedimented and discard the medium.
5. Add an appropriate volume of Enzyme Solution of Neuron Dissociation Solutions.

Note: The volumes of Neuron Dissociation Solutions are adjusted according to the stage and number of neocortices, as indicated in the following examples.

Stage (ICR)	Number of neocortices	Volume of solutions (μ l)
E8– E14	1 telencephalon or neocortex	50
E15–20 dpc	1 neocortex	100
E8–E12	20–30 neocortices (1 pregnant mouse)	100
E13–E16	20–30 neocortices (1 pregnant mouse)	200
E17–20 dpc	20–30 neocortices (1 pregnant mouse)	300

6. Incubate for 20 min in a heat block at 37°C.
7. Mix with a pipette (~20 times) attached to a P200 or P1000 Pipetman (Gilson).
8. Centrifuge at 600 \times g for 2 min at room temperature (18°C–24°C).

Note: For steps 8 and 10, centrifugation should be performed at room temperature (not 4°C), given that Neuron Dissociation Solutions may include DNase that is inactivated at 4°C.

9. Discard the supernatant, add Dispersion Solution to the pellet, resuspend the pellet with the use of a pipette, and add Isolation Solution below the Dispersion Solution.
10. Centrifuge at 600 \times g for 2 min at room temperature.
11. Discard the supernatant, add 200 μ l of 0.2% BSA/PBS to the pellet, and resuspend with a pipette.

Note: We remove debris with the use of Percoll solution at steps 12 to 15 for neocortices at E15 to 20 dpc, given that such debris can cause clogging of the FACS machine. We skip steps 12 to 15 for neocortices at earlier stages.

12. Prepare the following Percoll solution in another 1.5-mL tube.

Percoll solution		
Reagent	Final Concentration	Amount
Percoll	22.5% (v/v)	45 μ l
10 \times PBS		5 μ l
0.2% BSA/PBS		Up to 150 μ l
Total		200 μl

13. Layer cell suspension on top of Percoll solution.
14. Centrifuge at 600 \times g for 2 min at 4°C.
15. Discard the supernatant, add 200 μ l of 0.2% BSA/PBS to the pellet, and resuspend with a pipette.

Immunostaining

⌚ Timing: 20–30 min

16. Add fluorescent label–conjugated antibodies, such as phycoerythrin (PE)–conjugated anti-CD133, allophycocyanin (APC)–conjugated anti-CD133, and PE-conjugated anti-CD24 at a 1:400 dilution.

Note: Prepare negative control samples for immunostaining.

Note: For immunostaining of surface antigen on neural cells, [Menon et al. \(2014\)](#) provided a useful visual protocol ([Menon et al., 2014](#)).

17. Incubate for 10 min at 4°C.

Note: When staining with antibodies other than CD24 or CD133, it may be better to extend this time.

18. Centrifuge at 600 × *g* for 2 min at 4°C.
19. Discard the supernatant and add 300 μl of 0.2% BSA/PBS to the pellet for washing.
20. Centrifuge at 600 × *g* for 2 min at 4°C.
21. Discard the supernatant, add an appropriate volume of 0.2% BSA/PBS to the pellet, and resuspend with a pipette.
22. Filter and transfer the suspension using 5 mL Round Bottom Polystyrene Test Tube with Cell Strainer Snap Cap.

Note: We usually prepare the cell suspension at a density of ~ 1 × 10⁷ cells/mL or in a volume of >200 μl. Higher cell densities increase the speed of cell collection but result in a greater loss of marker-positive cells.

Cell sorting

⌚ Timing: 20–60 min

23. Run control samples, including non-stained and single-stained samples, in order to check for potential spectral overlap. If necessary, set up compensation.
24. Run fully stained samples and set gates. The following is an example for neocortical cells expressing GFP and stained with APC-conjugated anti-CD133 ([Figure 1](#)).
 - a. Remove debris according to the plot of forward scatter (FSC)–A and side scatter (SSC)–A.
 - b. Remove doublets according to plots of FSC–W and FSC–H and of SSC–W and SSC–H.
 - c. Separate GFP-positive (GFP^{pos}) and GFP-negative (GFP^{neg}) populations on the basis of FITC–A signals.
 - d. Determine cell populations—such as CD133^{high} cells using APC–A signals—with the use of GFP^{neg} cells.
 - e. Apply gates determined with the GFP^{neg} population to the GFP^{pos} population.

Note: For cells transgenic for *Nestin-d4Venus* or wild-type cells stained with PE-conjugated anti-CD133, high, medium, low, and negative fractions are determined as in [Figures 2A](#) and [2B](#).

25. Prepare 1.5-mL collection tubes.
 - a. Coat the inner wall of 1.5-mL tubes with 500 μl of 1% BSA/PBS.
 - b. Add 500 μl of 0.2% BSA/PBS to the coated 1.5-mL tubes as a cushion.

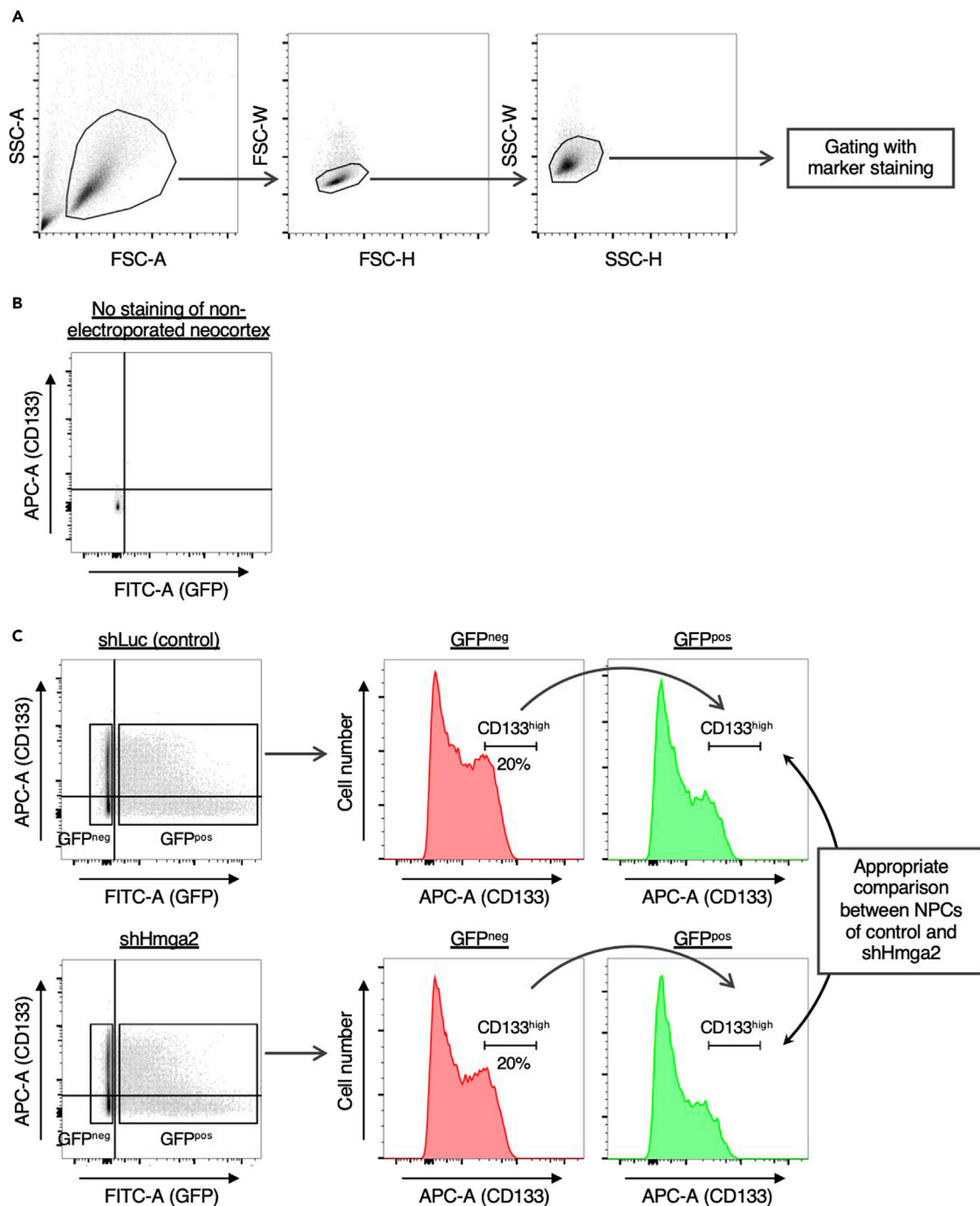


Figure 1. Gating strategies for isolation of CD133^{high} neural progenitors from the embryonic neocortex after in utero electroporation with a plasmid encoding GFP

Plasmids encoding GFP and either control (shLuc) or *Hmga2* (shHmga2) shRNAs were introduced into neural progenitors of mouse embryos at E11 by in utero electroporation as described previously (Sakai et al., 2019). Neocortical cells were dissociated at E13, stained with APC-conjugated antibodies to CD133, and subjected to FACS.

(A) Debris and doublets were removed as described in steps 24a and 24b.

(B and C) GFP and CD133 profiles of negative control (B) and electroporated and stained (C) samples. For electroporated samples, the CD133^{high} population (top 20% of CD133-expressing cells) was defined with the use of the GFP^{neg} fraction, and the gating was then applied to the GFP^{pos} fraction, as described in steps 24c to 24e. This approach allows appropriate comparison between neural progenitors of control and shHmga2 samples.

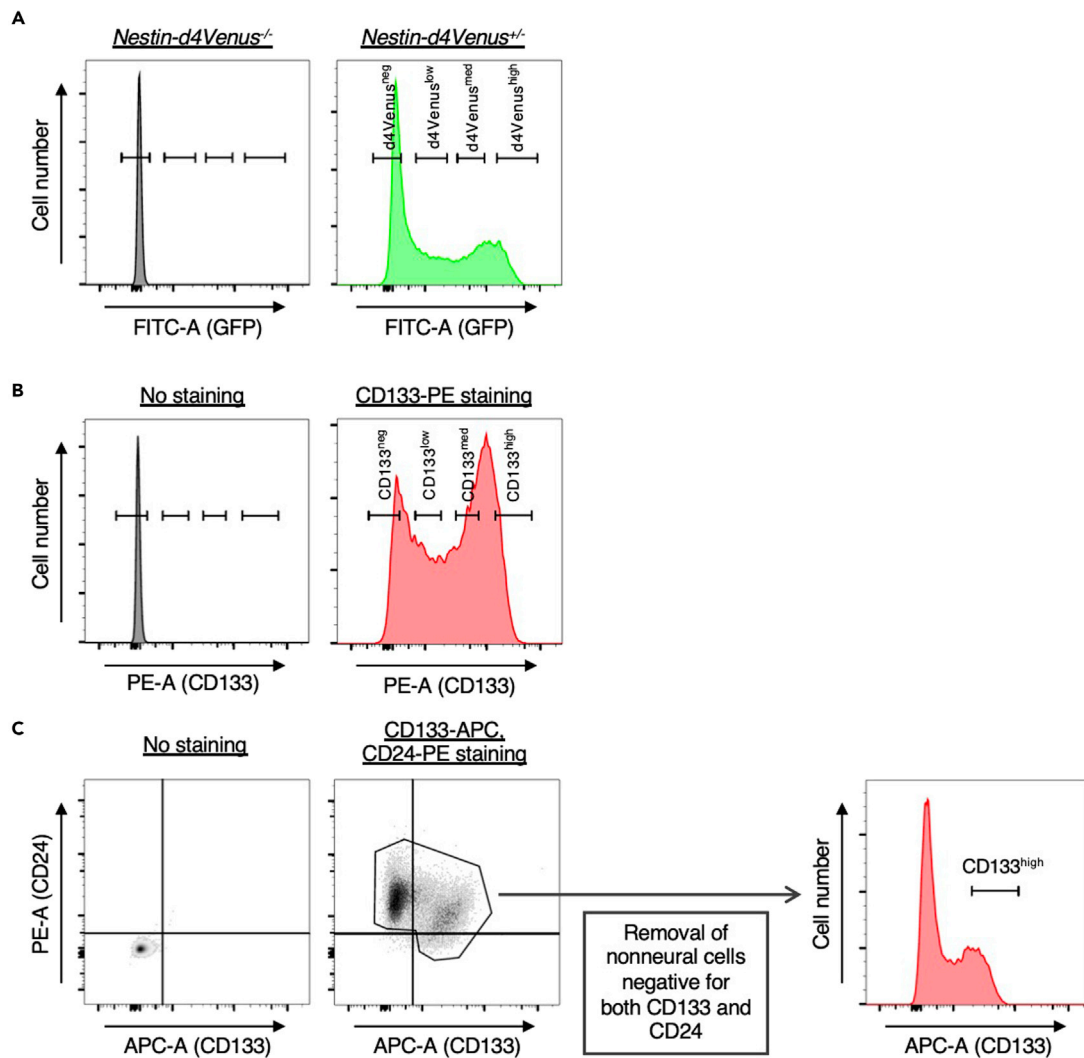


Figure 2. Gating strategies for isolation of neural progenitors and neurons from the neocortex of *Nestin-d4Venus* or wild-type embryos

(A) Neocortical cells of *Nestin-d4Venus^{-/-}* or *Nestin-d4Venus^{+/-}* embryos at E14 were dissociated and subjected to FACS analysis as described previously (Sakai et al., 2019). The gates for dVenus-high, -medium, -low, and -negative cells are shown.

(B) Neocortical cells of wild-type embryos at E14 were dissociated, stained (or not) with PE-conjugated antibodies to CD133, and subjected to FACS analysis as described previously (Sakai et al., 2019). The gates for CD133-high, -medium, -low, and -negative cells are shown.

(C) Neocortical cells of C57BL/6 embryos at E15 were dissociated, stained (or not) with both APC-conjugated antibodies to CD133 and PE-conjugated antibodies to CD24, and subjected to FACS analysis. Nonneural cells determined as those negative for both CD133 and CD24 were removed. The gate for CD133-high cells is shown.

26. Sort the populations of interest into the prepared 1.5-mL tubes.

Note: Given that the volume of one drop is ~4 nano liter for FACS Aria III and III with 100 μ m nozzle, we can collect 250,000 cells into one 1.5-mL tube.

27. Invert and mix each tube immediately after sorting. Given that the sheath solution—in this case, FACS Flow (BD)—may contain a detergent, its dilution by mixing with the cushion solution helps to preserve cell viability.

28. Centrifuge at 600 \times g for 5 min at 4°C.

29. Discard the supernatant.

30. For reverse transcription (RT) and qPCR and RNA-seq analyses, freeze the cell pellet with liquid N₂ and store at –80°C. 10,000 cells are enough for RT-qPCR and usual RNA-seq analyses, and 1,000 cells are used for Quartz-seq analysis (Eto et al., 2020; Kuwayama et al., 2020; Sakai et al., 2019).

▮▮ **Pause point:** Store the cell pellets at –80°C for more than 1 year.

Note: For other types of biochemical analysis, proceed to the appropriate treatment as follows.

- For chromatin immunoprecipitation (ChIP)-seq, crosslink the cell pellet using 1% formaldehyde solution for 10 min at room temperature. After centrifugation, washing the cell pellets with PBS, store at –80°C. We used 100,000 cells for H3K27me3 ChIP-seq (Kuwayama et al., 2020).
- For assay for transposase-accessible chromatin (ATAC)-seq, proceed to the ATAC reaction without freezing cells (Yamanaka et al., 2020). For freezing before ATAC reaction, cryopreservation can provide data with better quality (Milani et al., 2016). We used 20,000 cells for ATAC-seq (Yamanaka et al., 2020).
- For cleavage under targets and tagmentation (CUT&Tag), cryopreserve the cell pellet using cell stock solution, such as CELLBANKER (Takara). We can also directly sort the cells into the 1.5 mL tube, including cell stock solution in step 26. We used 10,000 cells for H3K27me3 and 100,000 cells for Ring1B CUT&Tag (Eto et al., 2020).

Checking the cell fractions by RT-qPCR

⌚ **Timing:** 1 day

- Extract total RNA from the frozen cells with the use of RNAiso Plus and measure its concentration with an absorption photometer such as NanoDrop.
- Subject the total RNA to RT with a ReverTra Ace qPCR RT Master Mix with gDNA Remover and dilute the generated cDNA 5- to 10-fold with water.

Note: We perform steps 31 and 32 under RNase-free conditions.

Note: 100 ng of total RNA are sufficient for the RT-qPCR analysis.

Alternatives: RNA extraction and RT can also be performed with other reagents or kits such as TRIzol (Thermo Fisher Scientific) and an RNeasy Mini Kit (Qiagen), respectively.

33. Prepare the following premix for qPCR.

Reagent	Final concentration	Amount
THUNDERBIRD SYBR qPCR Mix	50% (v/v)	2.5 μl/reaction
Forward primer (100 μM)	1.6 μM	0.08 μl
Reverse primer (100 μM)	1.6 μM	0.08 μl
ddH ₂ O		0.34 μl
Total		3 μl

⚠ **CRITICAL:** Given that we usually quantify the amount of target cDNA by generating a calibration curve and run a negative control sample consisting of water, it is necessary to make a premix for these samples.

34. Add 3 μl of the premix followed by 2 μl of diluted cDNA to 8-stripe tubes or a 96-well plate, mix, and centrifuge briefly.

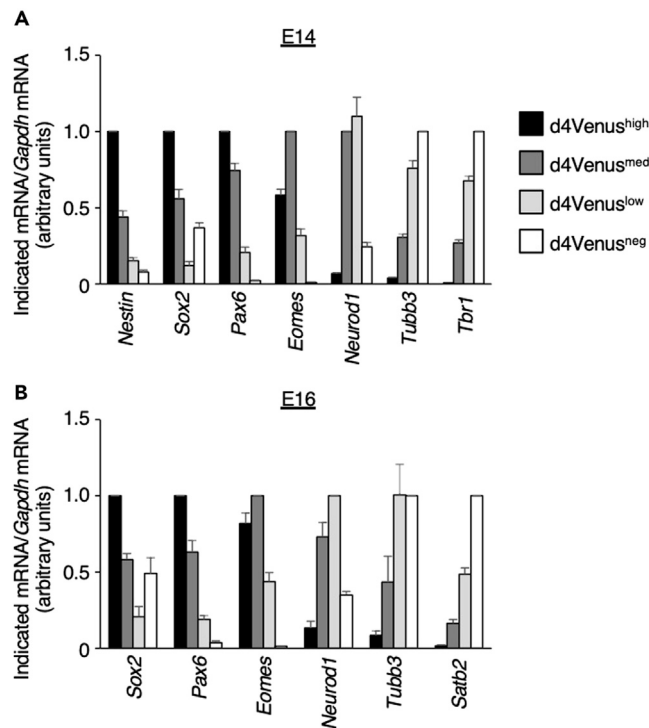


Figure 3. Quality check for neural progenitors and neurons isolated from the neocortex of *Nestin-d4Venus* embryos (A and B) Isolated d4Venus-high, -medium, -low, and -negative cells at E14 (A) or E16 (B) were subjected to RT-qPCR analysis of the indicated mRNAs. Data were normalized by the amount of *Gapdh* mRNA, expressed relative to the higher value for each mRNA, and are means + SEM from 11 (*Sox2*, *Pax6*, *Eomes*, *Neurod1*, *Tubb3*, and *Tbr1* at E14) and four (E16 and *Nestin* at E14) independent experiments independent experiments with the exception of *Nestin* mRNA (four independent experiments).

35. Perform the qPCR reaction in a LightCycler 480 instrument according to a protocol described by the manufacturer of the SYBR reagent (<https://www.toyobo-global.com/seihin/xr/lifescience/support/manual/QPS-201.pdf>).
36. Calculate the concentration of the target cDNA with LightCycler 480 software by absolute quantification.
 - △ **CRITICAL:** We always check that the calibration curve is appropriate, that the amplification efficiency is ~2.0, that the signal of the water sample is negligible, and that the melting temperature (T_m calling) curve has only one peak.
37. Calculate the expression levels of the target genes with normalization to an internal control gene such as *Gapdh*.

Note: *Actb* is another suitable internal control gene.

EXPECTED OUTCOMES

Successful cell sorting and RT-qPCR analysis should reveal the effect of an electroporated plasmid or changes in the expression of marker genes for neural progenitors or neurons at various differentiation stages. For example, introduction of a knockdown plasmid should result in an observed reduction in the amount of the target mRNA, such as was the case for knockdown of *Hmga2* mRNA in a previous study (Sakai et al., 2019). Examples of results for neural progenitors and neurons isolated from the *Nestin-d4Venus* mouse neocortex or from the wild-type mouse neocortex stained with anti-CD133 at E12, E14, E16, or E18 are shown in Figures 3 and 4. Marker genes for neural progenitors—such as *Nestin*,

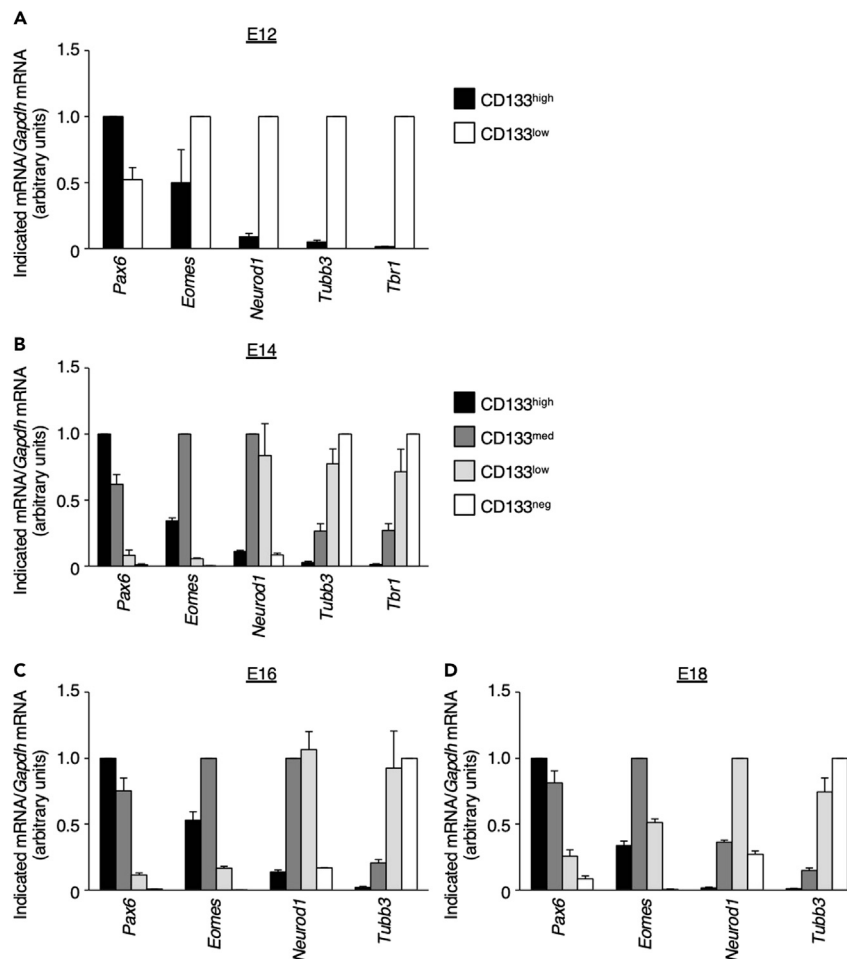


Figure 4. Quality check for neural progenitors and neurons isolated from the neocortex of wild-type embryos on the basis of CD133 staining

(A–D) Isolated CD133-high, -medium, -low, or -negative cells at E12 (A), E14 (B), E16 (C), or E18 (D) were subjected to RT-qPCR analysis of the indicated mRNAs. Data were normalized by the amount of *Gapdh* mRNA, are expressed relative to the higher value for each mRNA, and are means + SEM from three (E12, E18, and *Pax6* and *Tbr1* at E14) or four (E16 and *Eomes*, *Neurod1*, and *Tubb3* at E14) independent experiments.

Sox2, and *Pax6*—are highly expressed in d4Venus^{high} or CD133^{high} fractions; those for immature neurons, such as *Eomes* and *Neurod1*, are highly expressed in d4Venus-medium (d4Venus^{med}) and d4Venus^{low} fractions or in CD133^{med} and CD133^{low} fractions; and those for neurons—such as *Tubb3*, *Satb2*, and *Tbr1*—are highly expressed in d4Venus^{neg} or CD133^{neg} fractions.

LIMITATIONS

Neuronal differentiation is a multistep process. For example, immediately after neuronal fate commitment of neural progenitors, *Neurog1* and *Neurog2* are expressed, with this expression being followed by that of *Eomes* and subsequently by that of *Neurod1*. However, whereas cell sorting based on CD133 or on *Nestin-d4Venus* expression can distinguish between *Nestin*-positive neural progenitors and *Neurod1*-positive immature neurons, most *Neurog1*- or *Neurog2*-positive cells are present in the CD133^{high} fraction, and *Eomes*-positive cells are present in both CD133^{high} and CD133^{med} fractions (Figures 3 and 4). Separation of these cells thus requires the use of specific transgenic mice expressing a fluorescent protein under the control of promoters or enhancers of *Neurog1*, *Neurog2*, or *Eomes* (Arnold et al., 2009; Berger et al., 2004; Gong et al., 2003; Kara et al.,

2015). Intracellular immunostaining of crosslinked neural cells for Eomes/Tbr2, Satb2, Ctip2, and Tle4 is another option (Molyneaux et al., 2015; Sakib et al., 2021), although crosslinking restricts the types of subsequent experiments,

Given that mature neurons possess many processes, they are vulnerable to enzymatic dissociation and are easily killed. This protocol is therefore applicable only to cells whose differentiation from neural progenitors has been underway for a few days. We have found that neurons differentiated from neural progenitors that were electroporated at E12 can be recovered efficiently up to E16, but hardly at all after E17— even when the fluorescence of transfected cells was clearly observed with a fluorescence stereomicroscope before dissociation (data not shown). Deep-layer neurons that have been undergoing differentiation for >5 days thus cannot be recovered after dissociation. Other methods, such as isolation of labeled nuclei with transgenic mice or staining for a neuronal nuclear marker, such as NeuN (Mo et al., 2015; Rehen et al., 2005; Spalding et al., 2005), are necessary to recover mature neurons for biochemical experiments.

We have here described the isolation of the most differentiated neurons as d4Venus^{neg} or CD133^{neg} cells. However, these populations also include nonneural cells of the embryonic neocortex such as hematopoietic cells. Indeed, RNA-sequencing analysis of d4Venus-high, -medium, -low, and -negative fractions from *Nestin-d4Venus* embryos at E14 revealed that the d4Venus^{neg} population expressed hematopoietic genes at high levels (data not shown) (Sakai et al., 2019). For removal of such nonneural cells, staining of the differentiated neuronal marker CD24 together with that of CD133 is useful (Pruszek et al., 2009; Uchida et al., 2000). The nonneural cells are then eliminated as those negative for both CD24 and CD133 (Figure 2C). The CD133^{high} fraction also contains endothelial cells, which we can eliminate by discarding cells positive for isolectin B4 (IB4), a marker for endothelial cells.

TROUBLESHOOTING

Problem 1

Frequent clogging of the FACS machine at step 26

Potential solution

Most clogging of the FACS machine is caused by the stickiness of genomic material released from dead cells during cell dissociation and staining. We find that centrifugation through a Percoll solution is useful for removal of such material before cell sorting. If genomic material is still observed after resuspension of stained cells (step 21), it can be removed with a pipette tip. It is also important that the Neuron Dissociation Solutions are used at room temperature.

Problem 2

Taking much time for sorting at step 26

Potential solution

Generally, a shorter time for dissection, dissociation, immunostaining, and sorting provides better quality of the analysis. The dissection of the electroporated region under fluorescence stereomicroscope as noted at step 2 is valid for shortening the sorting time.

Problem 3

No obvious cell pellet after centrifugation at step 28

Potential solution

In general, it is possible to visualize a pellet containing >10,000 cells. Given that cells are readily trapped on the walls of tubes, coating of the inner surface of collection tubes with BSA before addition of the cushion buffer is necessary to ameliorate this problem. Although commercially available

low-binding tubes can be used for this purpose, coating with BSA is the best choice for prevention of cell trapping by the tube wall.

Problem 4

Low yield of RNA at step 31 or low signals in the other analysis than expected

Potential solution

In general, it is difficult to fully recover the cell number counted by the FACS machine after cell pelleting since the cells that die or become unhealthy during the sorting process cannot make the pellet by centrifugation. For reducing those cells, it is useful to mix the sheath and cushion solution immediately after sorting at step 27. It is also valid to count the cell number recovered after cell pelleting for checking recovering rate in each case.

Problem 5

Reads mapped to both genomic regions and electroporated plasmids after sequencing analysis

Potential solution

If electroporated plasmids contain the sequences from the mouse genome, corresponding reads are mapped to the genomic regions after RNA-seq, ChIP-seq, CUT&Tag, or ATAC-seq analyses and should be removed from the subsequent analysis.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yusuke Kishi (ykisi@mol.f.u-tokyo.ac.jp).

Materials availability

This study did not generate new unique materials.

Data and code availability

The data in this paper are available from the corresponding authors upon reasonable request.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.K. and Y.G.; methodology, Y.K.; investigation, Y.K.; writing, Y.K. and Y.G.; funding acquisition, Y.K. and Y.G.; supervision, Y.K. and Y.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

- Arnold, S.J., Sugnaseelan, J., Groszer, M., Srinivas, S., and Robertson, E.J. (2009). Generation and analysis of a mouse line harboring GFP in the *Eomes/Tbr2* locus. *Genesis* 47, 775–781. <https://doi.org/10.1002/dvg.20562>.
- Berger, J., Eckert, S., Scardigli, R., Guillemot, F., Gruss, P., and Stoykova, A. (2004). E1-Ngn2/Cre is a new line for regional activation of Cre recombinase in the developing CNS. *Genesis* 40, 195–199. <https://doi.org/10.1002/gene.20081>.
- Eto, H., Kishi, Y., Yakushiji-Kaminatsui, N., Sugishita, H., Utsunomiya, S., Koseki, H., and Gotoh, Y. (2020). The Polycomb group protein Ring1 regulates dorsoventral patterning of the mouse telencephalon. *Nat. Commun.* 11, 5709. <https://doi.org/10.1038/s41467-020-19556-5>.
- Gaiano, N., Kohtz, J.D., Turnbull, D.H., and Fishell, G. (1999). A method for rapid gain-of-function studies in the mouse embryonic nervous system. *Nat. Neurosci.* 2, 812–819. <https://doi.org/10.1038/12186>.
- Gong, S., Zheng, C., Doughty, M.L., Losos, K., Didkovsky, N., Schambra, U.B., Nowak, N.J., Joyner, A., Leblanc, G., Hatten, M.E., et al. (2003). A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. *Nature* 425, 917–925. <https://doi.org/10.1038/nature02033>.
- Kara, E.E., McKenzie, D.R., Bastow, C.R., Gregor, C.E., Fenix, K.A., Ogunniyi, A.D., Paton, J.C., Mack, M., Pombal, D.R., Seillet, C., et al. (2015). CCR2 defines in vivo development and homing of IL-23-driven GM-CSF-producing Th17 cells. *Nat. Commun.* 6, 8644–8717. <https://doi.org/10.1038/ncomms9644>.
- Kuwayama, N., Kishi, Y., Maeda, Y., Nishiumi, Y., Suzuki, Y., Koseki, H., Hirabayashi, Y., and Gotoh, Y. (2020). In utero gene transfer system for embryos before neural tube closure reveals a role for Hmga2 in the onset of neurogenesis. *bioRxiv* 125. 2020.05.14.086330. <https://doi.org/10.1101/2020.05.14.086330>.
- Matsui, A., Yoshida, A.C., Kubota, M., Ogawa, M., and Shimogori, T. (2011). Mouse in utero electroporation: controlled spatiotemporal gene transfection. *J. Vis. Exp.* <https://doi.org/10.3791/3024>.
- Menon, V., Thomas, R., Ghale, A.R., Reinhard, C., and Pruzak, J. (2014). Flow cytometry protocols for surface and intracellular antigen analyses of neural cell types. *J. Vis. Exp.* e52241. <https://doi.org/10.3791/52241>.
- Milani, P., Escalante-Chong, R., Shelley, B.C., Patel-Murray, N.L., Xin, X., Adam, M., Mandefro, B., Sareen, D., Svendsen, C.N., and Fraenkel, E. (2016). Cell freezing protocol suitable for ATAC-Seq on motor neurons derived from human induced pluripotent stem cells. *Sci. Rep.* 6, 25474. <https://doi.org/10.1038/srep25474>.
- Mo, A., Mukamel, E.A., Davis, F.P., Luo, C., Henry, G.L., Picard, S., Ulrich, M.A., Nery, J.R., Sejnowski, T.J., Lister, R., et al. (2015). Epigenomic signatures of neuronal diversity in the mammalian brain. *Neuron* 86, 1369–1384. <https://doi.org/10.1016/j.neuron.2015.05.018>.
- Molyneaux, B.J., Goff, L.A., Brettler, A.C., Chen, H.-H., Brown, J.R., Hrvatin, S., Rinn, J.L., and Arlotta, P. (2015). DeCoN: Genome-wide analysis of *in vivo* transcriptional dynamics during pyramidal neuron fate selection in neocortex. *Neuron* 85, 275–288. <https://doi.org/10.1016/j.neuron.2014.12.024>.
- Pruzak, J., Ludwig, W., Blak, A., Alavian, K., and Isacson, O. (2009). CD15, CD24, and CD29 define a surface biomarker code for neural lineage differentiation of stem cells. *Stem Cells* 27, 2928–2940. <https://doi.org/10.1002/stem.211>.
- Rehen, S.K., Yung, Y.C., McCreight, M.P., Kaushal, D., Yang, A.H., Almeida, B.S.V., Kingsbury, M.A., Cabral, K.M.S., McConnell, M.J., Anliker, B., et al. (2005). Constitutional aneuploidy in the normal human brain. *J. Neurosci.* 25, 2176–2180. <https://doi.org/10.1523/JNEUROSCI.4560-04.2005>.
- Sakai, H., Fujii, Y., Kuwayama, N., Kawaji, K., Gotoh, Y., and Kishi, Y. (2019). Plag1 regulates neuronal gene expression and neuronal differentiation of neocortical neural progenitor cells. *Genes Cells* 24, 650–666. <https://doi.org/10.1111/gtc.12718>.
- Sakib, M.S., Sokpor, G., Nguyen, H.P., Fischer, A., and Tuoc, T. (2021). Intracellular immunostaining-based FACS protocol from embryonic cortical tissue. *STAR Protocol.* 2, 100318. <https://doi.org/10.1016/j.xpro.2021.100318>.
- Spalding, K.L., Bhardwaj, R.D., Buchholz, B.A., Druid, H., and Frisén, J. (2005). Retrospective birth dating of cells in humans. *Cell* 122, 133–143. <https://doi.org/10.1016/j.cell.2005.04.028>.
- Sunabori, T., Tokunaga, A., Nagai, T., Sawamoto, K., Okabe, M., Miyawaki, A., Matsuzaki, Y., Miyata, T., and Okano, H. (2008). Cell-cycle-specific nestin expression coordinates with morphological changes in embryonic cortical neural progenitors. *J. Cell Sci.* 121, 1204–1212. <https://doi.org/10.1242/jcs.025064>.
- Tabata, H., and Nakajima, K. (2001). Efficient in utero gene transfer system to the developing mouse brain using electroporation: visualization of neuronal migration in the developing cortex. *Neuroscience* 103, 865–872.
- Tsuboi, M., Kishi, Y., Yokozeki, W., Koseki, H., Hirabayashi, Y., and Gotoh, Y. (2018). Ubiquitination-Independent repression of PRC1 targets during neuronal fate restriction in the developing mouse neocortex. *Dev. Cell* 47, 758–772.e5. <https://doi.org/10.1016/j.devcel.2018.11.018>.
- Uchida, N., Buck, D.W., He, D., Reitsma, M.J., Masek, M., Phan, T.V., Tsukamoto, A.S., Gage, F.H., and Weissman, I.L. (2000). Direct isolation of human central nervous system stem cells. *Proceedings of the National Academy of Sciences of the United States of America* 97, 14720–14725. <https://doi.org/10.1073/pnas.97.26.14720>.
- Viesselmann, C., Ballweg, J., Lombard, D., and Dent, E.W. (2011). Nucleofection and primary culture of embryonic mouse hippocampal and cortical neurons. *J. Vis. Exp.* e2373. <https://doi.org/10.3791/2373>.
- Yamanaka, S., Kishi, Y., and Siomi, H. (2020). ATAC-seq method applied to embryonic germ cells and neural stem cells from mouse: Practical tips and modifications. *Epigenet. Methods* 128, 371–386. <https://doi.org/10.1016/B978-0-12-819414-0.00018-5>.