Prospective isolation of adult neural stem cells from the mouse subependymal zone

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Neural stem cells (NSCs) have the remarkable capacity to self-renew and the lifelong ability to generate neurons in the adult mammalian brain. However, the molecular and cellular mechanisms contributing to these behaviors are still not understood. Now that prospective isolation of the NSCs has become feasible, these mechanisms can be studied. Here we describe a protocol for the efficient isolation of adult NSCs, by the application of a dual-labeling strategy on the basis of their glial identity and ciliated nature. The cells are isolated from the lateral ventricular subependymal zone (SEZ) of adult hGFAP-eGFP (human glial fibrillary acidic protein–enhanced green fluorescent protein) transgenic mice by fluorescence-activated cell sorting. Staining against prominin1 (CD133) allows the isolation of the NSCs (hGFAP-eGFP$^+$/prominin1$^+$), which can be further subdivided by labeling with the fluorescent epidermal growth factor. This protocol, which can be completed in 7 h, allows the assessment of quantitative changes in SEZ NSCs and the examination of their molecular and functional characteristics.

INTRODUCTION

Adult neurogenesis persists only in a few niches within the mammalian brain, in which specialized glial cells, the adult NSCs, generate new neurons throughout life. In rodents, the major sites of adult neurogenesis are the SEZ of the lateral ventricles (**Fig. 1a**), which generates interneurons of the olfactory bulb, and the subgranular zone of the dentate gyrus in the hippocampus, which generates granule neurons (for reviews, see refs. 1,2). As equivalent regions appear to also exist in humans (as reviewed in ref. 3), this increases the potential for using these endogenous NSCs for repair. Interestingly, NSCs and/or their progeny seemingly react to adjacent brain injury and have the potential to generate a variety of different neurons, depending on the site of the injury[4–7]. Moreover, after demyelinating injury the SEZ also reacts by increasing the generation of progenitors for myelinating oligodendrocytes that are migrating to the site of injury[8]. Thus, cells from this niche may indeed provide an exciting source for endogenous repair in the adult mammalian brain. However, the molecular mechanisms governing adult stem and progenitor cells and their reaction to injury remain largely unknown.

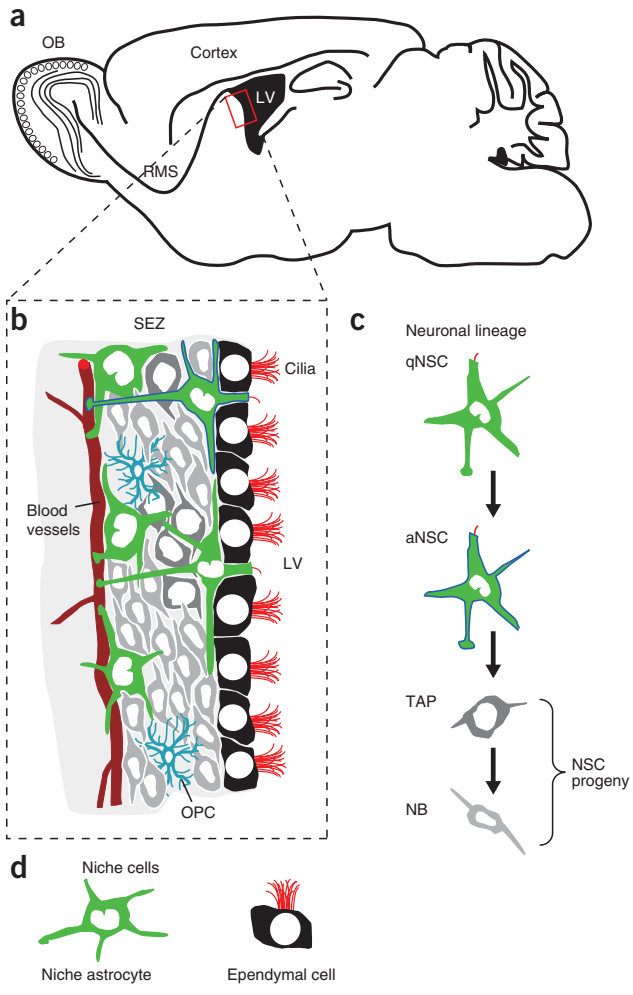

Figure 1 | Major cell types of the adult SEZ. (**a**,**b**) Schematic drawing of a sagittal section through an adult mouse brain. Red delineates the field of SEZ shown in the schematic drawing in **b**. (**b**) Simplified scheme depicting the cellular composition of adult SEZ. (**c**,**d**) Individual cells in the neurogenic lineage (**c**) or neurogenic niche (**d**). A small apical cilium (prominin1$^+$; red) of a hGFAP-eGFP$^+$ (green) adult neural stem cell contacts the ventricle, whereas basal end-feet contact the blood vessels[31,32]. The expression of EGF receptor (blue line) has been suggested to mark activated neural stem cells (aNSCs), whereas quiescent neural stem cells (qNSCs) should not express EGFR[17]. Multiciliated ependymal cells are directly in contact with the liquid-filled ventricle and express prominin1. Niche astrocytes are located more basally in the SEZ and express hGFAP-eGFP. Cortex, cerebral cortex; LV, lateral ventricle; NB, neuroblast; OB, olfactory bulb; OPC, oligodendrocyte progenitor; RMS, rostral migratory stream; TAP, transient amplifying progenitor cell.

One reason for this lack of information has been the absence of a method to prospectively isolate NSCs at sufficient purity for molecular analysis. However, this hurdle has recently been overcome by a dual-marker strategy[9]. The same approach that was successful for isolating radial glial cells in development[10] (a combination of cell-surface labeling for prominin1 (ref. 11), which is located on cilia (**Fig. 1b–d**)[12], and fluorescence of eGFP driven by the human GFAP promoter[13]) has now also allowed for the purification of adult NSCs without culturing, yielding unprecedented purity[9]. Indeed, lineage tracing by the novel split-Cre technology, following the progeny of cells coexpressing hGFAP and prominin1, further confirmed the long-term self-renewing and neurogenic capacity of these cells *in vivo*[9].

Several attempts have been made to enrich the adult NSC population from the SEZ by fluorescence-activated cell sorting (FACS), but purification was either below 35% (refs. 14–16) or the isolated fraction contained only a proportion of the stem cells[17,18]. Notably, some of the antigens that were previously used to enrich neurosphere-forming cells, such as CD133 (1.5% neurosphere formation[15]) or the carbohydrate Lewis X (recognized by CD15 antibodies[14]), overlap with the hGFAP-eGFP/prominin1 double-positive cells, but they have not been sufficiently selective on their own. Another transgenic mouse line expressing GFP rather than eGFP under the hGFAP promoter (thereby resulting in weaker fluorescence levels) has been combined with the expression of the epidermal growth factor (EGF) receptor and resulted in 30% neurosphere-forming cells[17]. Other purification protocols involved *in vitro* expansion of progenitor cells (often isolated from embryonic or postnatal tissues), and treatment with growth factors, which profoundly alters the gene expression pattern and behavior of cells, thereby rendering them rather different from their *in vivo* counterparts[19–23].

We present here a fast isolation protocol (~7 h) that directly labels NSCs, niche astrocytes and ependymal cells, and that allows for highly enriching these cell fractions from the adult SEZ without the need for *in vitro* amplification or any further treatment of the cells, such as the addition of growth factors. Therefore, the isolated cells are as close to the *in vivo* situation as possible and can be used for mRNA profiling[9], cell culture followed by live imaging[23,24], or for quantification in various mutant backgrounds or upon specific treatments. Moreover, this protocol can be further refined by combination with previous protocols that use the fluorescent ligand of the EGF receptor to label the EGFR$^+$ cells[17,23,25]. This allows for further subdividing the NCS population into EGFR$^+$/hGFAP-eGFP$^+$/prominin1$^+$ and EGFR$^-$/hGFAP-eGFP$^+$/prominin1$^+$ cells, which most likely correspond to the fraction of activated and quiescent NSCs, respectively, as has been previously linked to the presence and absence of the EGFR[17,23]. A flow chart of the procedure can be found in **Figure 2**.

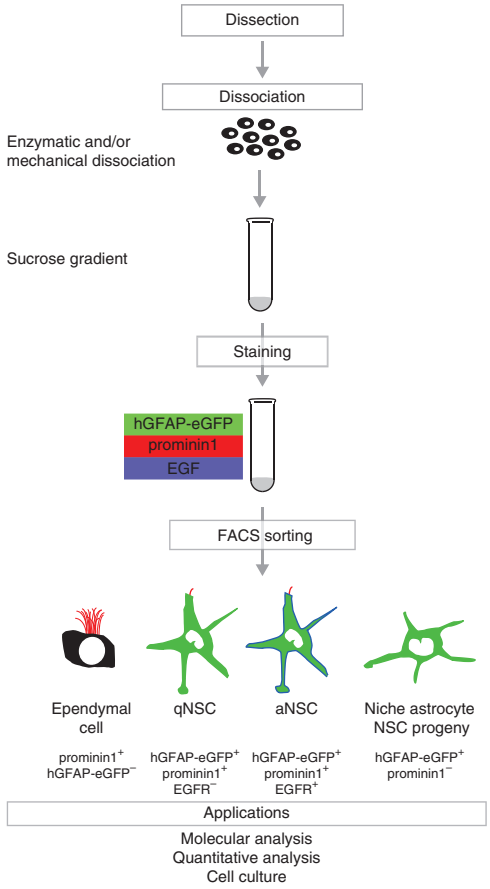

Figure 2 | Flow diagram depicting the major steps of the isolation protocol and possible further applications.

This protocol offers the most efficient method of isolation of NSCs, niche astrocytes and ependymal cells from the adult mouse SEZ. However, one major limitation of the protocol is the necessity of using transgenic hGFAP-eGFP mice[13].

MATERIALS

REAGENTS

▲ **CRITICAL** We recommend that all reagents be sterile to avoid contamination of the FACS instrument. However, the protocol also works with nonsterile reagents and the decision should be made depending on the final application.

- BSA (Sigma-Aldrich, cat. no. A9418)
- Dulbecco's PBS without $CaCl_2$ and $MgCl_2$ (D-PBS, 1×; Invitrogen, cat. no. 14190)
- Earle's Balanced Salt Solution (EBSS, 1×; Invitrogen, cat. no. 24010)
- Hank's Balanced Salt Solution with $CaCl_2$ and $MgCl_2$ (HBSS, 1×; Invitrogen, cat. no. 24020)
- Heat-inactivated FBS (PAN, cat. no. 3302-P102305) ▲ **CRITICAL** Inactivate the serum by incubation at 56 °C for 30 min, prepare aliquots and store at −20 °C for up to 6 months.
- D-(+)-Glucose solution (45% (wt/vol) in H_2O; Sigma-Aldrich, cat. no. G8769)
- HEPES buffer solution (1 M; Invitrogen, cat. no. 15630)
- D-(+)-Saccharose (Carl Roth, cat. no. 9097.2)
- Sodium azide (Merck, cat. no. 106688) ! **CAUTION** Sodium azide is very toxic. Avoid inhalation, ingestion and skin contact. Work under a hood. Prepare a 1% (wt/vol) stock solution by adding 0.1*g* sodium azide to 10 ml water and store at 4 °C for up to 2 months.
- Sodium bicarbonate (7.5% (wt/vol); Invitrogen, cat. no. 25080060)
- Trypsin (0.05% (wt/vol)) with EDTA (Invitrogen, cat. no. 25300)
- Antibody for FACS: CD133-PE (prominin1; eBioscience, cat. no. 12-1331)
- Isotype control: rat IgG1 K isotype control phycoerythrin (PE) (eBioscience, cat. no. 12-4301)
- Fluorescent ligand of EGF receptor for FACS: EGF–Alexa Fluor 647 (Molecular Probes, cat. no. E-35351) ▲ **CRITICAL** Store antibody, isotype control and ligand protected from light.
- Propidium iodide (PI, Sigma-Aldrich, cat. no. P4170) ! **CAUTION** PI can cause irritation to eyes, skin and the respiratory tract.
- FACS buffer: FACSFlow sheath fluid (BD Biosciences, cat. no. 342003)
- Heterozygous hGFAP-eGFP mice[13]

• C57BL/6J wild-type (WT) mice ▲ CRITICAL We have tested the sorting protocol for animals of different ages. As stem and progenitor cell composition changes with age, be aware that the proportions described in ref. 9 were obtained with 2- to 3-month-old mice. For analysis of mutants or other experimental treatments please ensure the use of age-matched animals. ▲ CRITICAL For the protocol described here, it is important to use the mouse line described originally in ref. 13 and maintain it on the original background rather than backcrossing to C57BL/6J, which results in variegation. ! CAUTION All experiments should be performed in accordance with all relevant governmental and institutional regulations regarding the use of animals for research purposes.

EQUIPMENT

Microdissection instruments

• Dumont no. 5SF forceps, Inox super fine tip (Fine Science Tools, cat. no. 11252-00)
• Dumont no. 7 forceps, Dumostar (Fine Science Tools, cat. no. 11297-00)
• Spring scissors, Vannas-Tubingen (5 mm; Fine Science Tools, cat. no. 15003-08)
• Surgical disposable scalpels (B. Braun, cat. no. 5518083)
• Surgical scissors, Metzenbaum (18 cm; Fine Science Tools, cat. no. 14016-14)

Cell culture

• Tissue culture dishes (60 mm; Greiner Bio-One, cat. no. 633180)
• Conical tubes (15 ml; BD, cat. no. 352096)
• Conical tubes (50 ml; BD, cat. no. 352070)
• Polypropylene round-bottom tubes (5 ml, FACS tubes; BD Biosciences, cat. no. 352063)
• Cell strainer (70 μm; BD, cat. no. 352350)
• Glass Pasteur pipettes (150 mm; Fisherbrand, cat. no. FB50251; sterilized using autoclave)
• Bottle-top filter system (0.22 μm, 500 ml; TPP, cat. no. 99500)
• Centrifuge suitable for 15 and 50 ml tubes with 4 °C cooling option (Rotanta 460R; Hettich Lab Technology)
• Water bath at 37 °C
• Laminar flow hood
• Dissecting microscope (Leica)

FACS

• Flow cytometry cell sorter: FACSAria I with FACSDiva software (BD Biosciences)
• Cover slips, coated with poly-D-lysine.

REAGENT SETUP

Propidium iodide Prepare a 1 mg ml^{-1} stock solution by adding 1 mg of PI to 1 ml of water and store it at 4 °C protected from light for up to 6 months. ! CAUTION PI can cause irritation to eyes, skin and the respiratory tract.

Dissection medium Prepare dissection medium by adding 5 ml of HEPES 1 M (final concentration 10 mM) to 500 ml of HBSS 1×. Store the dissection medium at 4 °C for up to 2 weeks.

Dissociation solution To prepare 5 ml of dissociation solution, pipette 100 μl of 0.05% (wt/vol) trypsin (final concentration 0.001% (wt/vol)) to 5 ml of Solution I. ▲ CRITICAL This solution must be prepared shortly before use. ! CAUTION Be aware that enzymatic digestion (here with trypsin) digests cell-surface proteins. We have examined prominin1 and EGFR labeling with and without trypsin, and found no difference in the proportion of labeled cells. However, this may be different for other proteins, should you wish to examine the cell types for other cell-surface proteins. Moreover, you may want to test different enzymes to further improve cell survival, especially if you aim to culture them thereafter. We have tested papain (Worthington) and Accutase (Millipore) and found no substantial difference in the proportion of PI-labeled cells (i.e., in the rate of cell death).

Solution I (HBSS-glucose) Prepare 500 ml of solution I by adding 50 ml of 10× HBSS, 9 ml of 45% (wt/vol) D-(+)-glucose (final concentration 0.81%) and 7.5 ml of 1 M HEPES (final concentration 15 mM) to 433.5 ml of ddH_2O. Adjust the pH to 7.5 using 7.5% (wt/vol) sodium bicarbonate. Sterilize the solution by filtration (0.22-μm filter) and prepare appropriate aliquots. Store at −20 °C for up to 6 months.

Solution II (saccharose-HBSS) To prepare 500 ml of solution II, dissolve 154 g of D-(+)-saccharose (final concentration 0.9 M) in 25 ml of 10× HBSS; add ddH_2O to a final volume of 500 ml. Adjust the pH to 7.5 using 7.5% (wt/vol) sodium bicarbonate. Sterilize the solution by filtration (0.22-μm filter) and prepare appropriate aliquots. Store at −20 °C for up to 6 months.

Solution III (BSA-EBSS-HEPES) To prepare 500 ml of solution III, add 10 ml of 1 M HEPES (final concentration 20 mM), 20 g of BSA, and fill up to a final volume of 500 ml with EBSS. Adjust the pH to 7.5 using 7.5% (wt/vol) sodium bicarbonate. Sterilize the solution by filtration (0.22-μm filter) and prepare appropriate aliquots. Store at −20 °C for up to 6 months.

Staining solution Add 200 μl of 1% (wt/vol) sodium azide solution (final concentration 0.02%, wt/vol) and 1 ml of FBS (final concentration 10%, vol/vol) to 10 ml of PBS. ! CAUTION Always freshly prepare the staining solution. ! CAUTION Sodium azide is very toxic. Avoid inhalation, ingestion and skin contact.

EQUIPMENT SETUP

FACS setup Start the FACS instrument and, if required, sterilize the tubing by running 70% (vol/vol) ethanol for 15 min as sheath fluid. Then run normal sheath fluid for at least 10 min. ! CAUTION Always use filtered solutions to avoid the presence of any crystals. ! CAUTION Adjust sheath fluid to your needs. Some sheath fluids contain preservatives, which may be toxic for the cells (note that sheath fluid and sample fluid mix in the sample collection chamber). We have tested FACSFlow sheath fluid, which was compatible with the isolation of SEZ cells followed by culturing over a long time, but survival rates may be even higher if you use sterile PBS. ! CAUTION Depending on the downstream applications, consider which nozzle to use. For most experiments, we have used a 70-μm nozzle with an appropriate sheath pressure of 70 p.s.i. We have also tested a 100-μm nozzle at 20 p.s.i. and found no obvious difference. However, cell viability for long-term culturing may be further improved by using the 100-μm nozzle.

PROCEDURE

Dissection of the SEZ ● TIMING ~15 min per mouse

1| Euthanize the animals according to the approved procedure at your institution and carefully dissect the brain (**Fig. 3**). (Animals may be killed by cervical dislocation, in rising CO_2 or under anesthesia, depending on the relevant regulations for the use of animals.)

! CAUTION Handling of experimental animals must be performed in accordance with the relevant governmental and institutional regulations regarding the use of animals for research purposes.

2| Transfer the brain into a culture dish containing 10 ml of ice-cold dissection medium. Dissection should be done under the microscope.

▲ CRITICAL STEP Keep brains in dissection medium on ice during the dissection period.

3| Make a coronal cut through the optic chiasm to separate forebrain from midbrain (**Fig. 3a**).

4| Divide the two hemispheres by cutting the brain along the midline (**Fig. 3a**,**b**). The hippocampus can now be seen in the cross-sections (**Fig. 3c**).

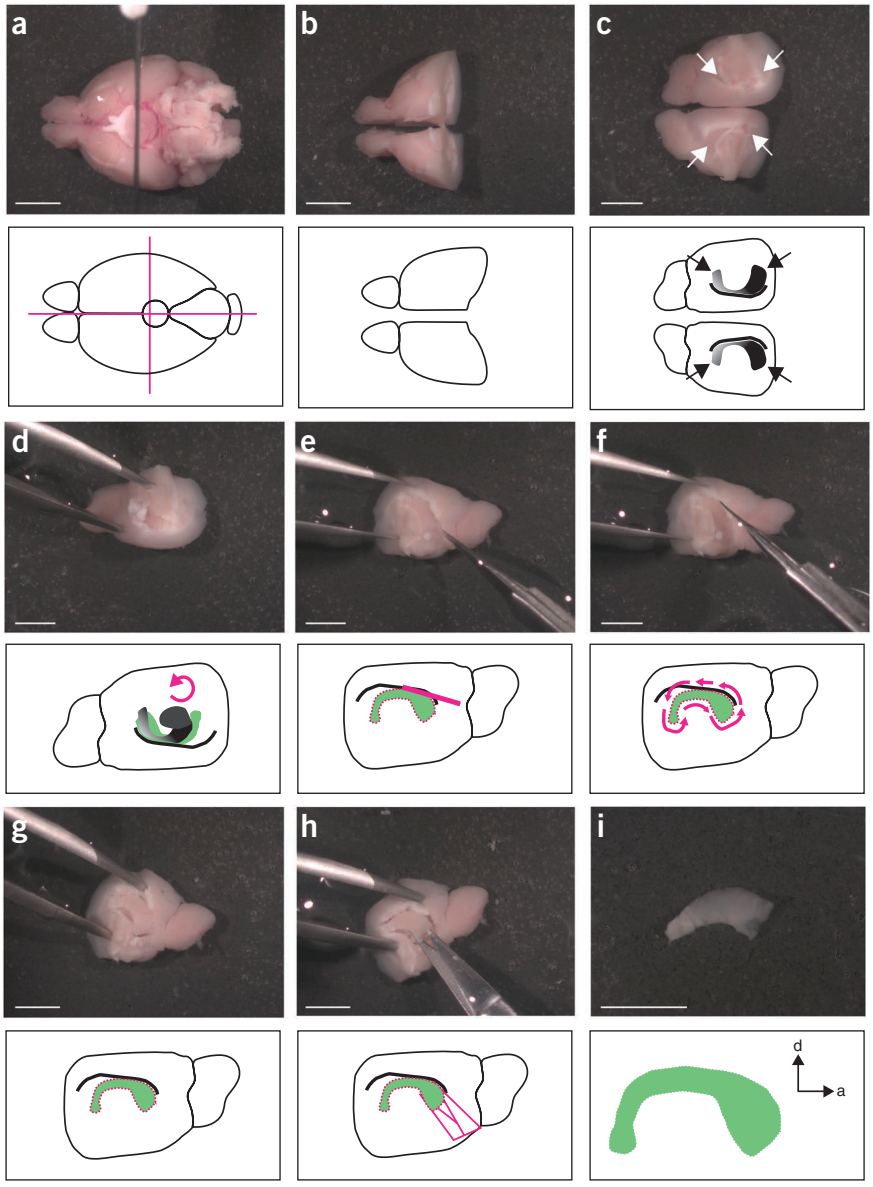

Figure 3 | Dissection procedure of the lateral ventricular wall. (**a**,**b**) Photographs and schemes depicting the position of the transverse (at the level of the optic chiasm) and longitudinal (along the midline) cut (**a**) to isolate the anterior part of the forebrain hemispheres (**b**). (**c**) Medial view of both hemispheres with arrows pointing to rostral and caudal ends of the hippocampus. (**d**) Image and scheme depicting the removal of the hippocampus in order to uncover the underlying lateral wall of the lateral ventricle. (**e**–**h**) Images and schemes demonstrating the dissection of the SEZ from the surrounding white matter (**f**) and striatum (**h**). (**i**) Image of the isolated SEZ. Scale bar, 5 mm. For a similar dissection protocol, see also refs. 24,33.

5| Insert a forceps between the hippocampus and the white matter of the cortex, and then carefully loosen these from each other to remove the hippocampus from the brain (**Fig. 3d**).
! CAUTION The lateral wall of the lateral ventricle is located directly underneath the hippocampus. While removing the hippocampus, be careful not to insert the forceps too deeply, thereby damaging the SEZ.

6| On removal of the hippocampus and the medial ventricular wall, you will look directly onto the lateral wall of the ventricle, which is visible by the striped pattern appearance due to the underlying striatum. Cut around the SEZ using the Vannas-Tubingen spring scissors, following its banana-like shape (**Fig. 3e**,**f**).

7| Isolate the excised lateral wall by inserting the scissors between SEZ and the underlying striatum to carefully cut and therefore separate them from each other (**Fig. 3g**,**h**).
▲ CRITICAL STEP Keep dissected SEZs in the dissection medium on ice.
▲ CRITICAL STEP It is important to cut as thinly as possible to avoid the underlying striatal tissue.

Dissociation and purification ● TIMING ~1.5 h
8| Transfer the SEZs into a 15-ml conical tube containing 5 ml of solution I and mechanically dissociate the tissue by pipetting up and down ten times with a fire-polished glass Pasteur pipette that has been precoated with 10% (vol/vol) FBS in PBS.
! CAUTION Avoid generating air bubbles when triturating the tissue, as this will reduce the viability of the cells.
! CAUTION This and subsequent steps are optimized for SEZ preparation of up to six mice. If you plan using SEZs of more mice, we recommend splitting the samples.
? TROUBLESHOOTING

9| Add 100 µl of 0.05% (wt/vol) trypsin to the sample and incubate the cell suspension at 37 °C for 15 min. After 15 min, remove the tube from the water bath and triturate ten times with a fire-polished and coated Pasteur pipette; incubate the cell suspension for an additional 15 min. Triturate the tissue ten times at the end of the incubation.
! CAUTION Do not incubate longer than 30 min, as this will decrease the viability of the cells.

▲ CRITICAL STEP It is important to dissociate the SEZ to single cells, as any remaining aggregates are removed later by filtration, which can result in the loss of many cells.
? TROUBLESHOOTING

10| Add 5 ml of ice-cold solution III and mix solutions by pipetting several times up and down. The BSA in solution III stops the activity of the trypsin.
! CAUTION Avoid generating air bubbles, as this will reduce the viability of the cells.
? TROUBLESHOOTING

11| Place a 70-μm cell strainer into a 50-ml conical tube. Pass cells through the strainer to remove aggregates and then transfer the filtrate back into a 15-ml conical tube. Centrifuge at 180*g* for 5 min at 4 °C.
▲ CRITICAL STEP Cell straining is important to remove cell aggregates for FACS analysis. Cell aggregates can clog the nozzle.
? TROUBLESHOOTING

12| Aspirate the supernatant carefully and resuspend the cells in 10 ml of ice-cold solution II by pipetting up and down. Centrifuge at 510*g* for 20 min at 4 °C.

13| Aspirate the supernatant carefully and resuspend the cells in 2 ml of ice-cold solution III. Prepare a 15-ml conical tube containing 12 ml of ice-cold solution III and add the 2-ml cell suspension on top. Centrifuge at 290*g* for 12 min at 4 °C.

Staining for FACS ● TIMING ~50 min
14| Aspirate the supernatant and resuspend the pellet in staining solution. Distribute the hGFAP-eGFP cell suspension into three tubes. Stain the cells with antibody, ligand and isotype control as follows (**Fig. 2**): for tube 1, stain the cells from hGFAP-eGFP mice with CD133-PE (1:100) and EGF–Alexa Fluor 647 (1:100) in staining solution. For tube 2, stain the cells from hGFAP-eGFP with rat IgG1 K isotype control PE (1:100) in staining solution. For tube 3, add cells from WT mice to staining solution lacking PI and any antibody or fluorescent ligand. For tube 4, incubate hGFAP-eGFP samples in staining solution lacking PI and any antibody or fluorescent ligand. Incubate for 30 min at 4 °C in the dark.
! CAUTION In the following steps, cells from WT (GFP-negative) animals need to be included as controls for setting gates later on in FACS analysis (**Fig. 4a**,**e**,**f**).
▲ CRITICAL STEP Keep samples chilled and protected from light until FACS analysis is carried out.
▲ CRITICAL STEP For proper analysis, all samples must be treated exactly the same way.
? TROUBLESHOOTING

15| After incubation, add 1 ml of staining solution to tubes 1–4 and centrifuge at 130*g* for 5 min at 4 °C. Aspirate supernatants carefully and wash the cells with 1 ml of staining solution.

16| Aspirate supernatants and resuspend in 500 μl of staining solution. Transfer all samples through a cell strainer (70 μm) (to avoid clumps of cells, which may have formed) to FACS tubes and protect from light.
! CAUTION Cells stick to plastic, so either coat the tubes with 10% (vol/vol) FBS in PBS or use polypropylene tubes.

17| Immediately before FACS analysis, add PI to tube 4 (1:1,000; final concentration, 1 μg ml^{-1}) and incubate it for 10 min at 4 °C to determine the proportion of dying cells (**Fig. 4a**,**b**).
? TROUBLESHOOTING

FACS analysis and sorting ● TIMING ~2–3 h
18| Vortex the samples before placing the tubes into the FACS instrument. To analyze and sort the cells, adjust the gates in the forward scatter–area (FSC-A) and the side scatter–area (SSC-A) to exclude cell debris (**Fig. 4c**) and include cells of interest (**Fig. 4c** and **Supplementary Fig. 1**); set the gates in FSC-A and forward scatter–width (FSC-W) to exclude cell aggregates (**Fig. 4d**). Analyze the following control samples: tube 3 (WT cells unstained as control for transgenic hGFAP-eGFP cells and $EGFR^+$ and PI^+ cells; see **Figs. 4** and **5**); tube 4 (cells stained with PI to analyze the rate of cell death (see Step 17), **Fig. 4b**); and tube 2 (rat IgG1 K isotype control PE, as an isotype control for prominin1$^+$ cells; **Figs. 4** and **5**). Determine the rate of cell death by measuring the proportion of PI^+ cells using tube 4 (**Fig. 4a**,**b**). Discard all experiments with cell death rates higher than 5%. If your observed rate of cell survival is acceptable, use FSC-A and SSC-A to exclude cell debris (low in FSC and SSC) as depicted in **Figure 4c**.
▲ CRITICAL STEP We confirmed the setup for the PI gate and the amount of dead cells by sorting PI^+ and PI^- cells and staining them with trypan blue as a marker of cell viability.
! CAUTION To exclude cell doublets, carefully adjust the gates for FSC-A and FSC-W (**Fig. 4d**). As a readout, plate the cells after sorting and determine the number of doublets within the sorted population.
! CAUTION Compensation needs to be performed for PE and fluorescein isothiocyanate (FITC) when both are excited with a blue laser.

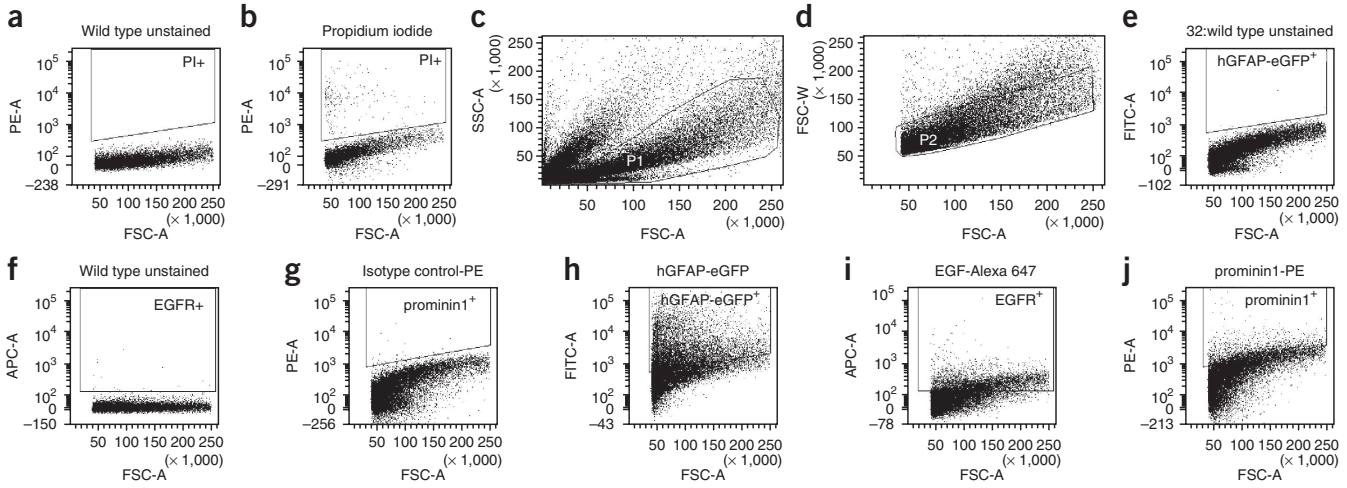

Figure 4 | FACS plots for gate setting for the different marker analysis. (**a**,**b**) Assessment of the dead cells in the sample determined by propidium iodide (PI) labeling. (**a**) Dot plot depicting definition of PI gate according to sample without PI. (**b**) Dot plot of representative sample containing <5% of dying cells. If the percentage of dead cells in the SEZ sample exceeds 5%, the sample should not be further analyzed. (**c**–**g**) Dot plots depicting the gate settings for different markers used to isolate the distinct SEZ cell types by FACS. (**c**) Select relevant and living cells by FCS-A and SSC-A (P1). (**d**) Cellular aggregates are excluded based on FSC-A and FSC-W gate (P2). (**e**,**f**) Gates for hGFAP-eGFP sorting (**e**) and EGF-Alexa Fluor 647–streptavidin complex (EGF-Alexa Fluor 647; **f**) were based on wild-type mice, which are negative for GFP and not incubated with a fluorescent ligand. (**g**) The gate for prominin1-PE (PE-conjugated CD133) was based on isotype-matched antibody control conjugated to PE. (**h**–**j**) Dot plots depicting cells positive for hGFAP-eGFP (**h**), EGFR (**i**) and prominin1 (**j**). FACS data are reported as suggested by Alexander *et al.*[34]. APC, Allophycocyanin.

19| Set the gates for hGFAP-eGFP and the EGF receptor ligand conjugated to Alexa Fluor 647 by using WT unstained cells (tube 3) and the isotype-matched antibody control conjugated to PE for prominin1-PE (tube 2; **Fig. 5a–c**). Then, using tube 1, sort the quiescent NSCs (hGFAP-eGFP$^+$, prominin1$^+$, EGFR$^-$), activated NSCs (hGFAP-eGFP$^+$, prominin1$^+$, EGFR$^+$), niche astrocytes (hGFAP-eGFP$^+$ only) and ependymal cells (prominin1$^+$ only) simultaneously (**Fig. 5d,e**). Collect the cells directly into FACS tubes suitable for sorting (nonadherent or coated), containing 1–2 ml of culture medium or buffers, depending on further applications (**Fig. 2**). By using the gate settings described above and the dissection procedure depicted in **Figure 3** in about 2- to 3-month-old mice, we observed, on average, that 20% of single, living cells were hGFAP-eGFP$^+$ (heterozygous transgenic animals), 9% were prominin1$^+$ and about 18% of cells were labeled with EGF-Alexa Fluor 647. The proportion of hGFAP-eGFP$^+$/prominin1$^+$ double-positive cells is about 2.5%.

! CAUTION The purity of the sorted sample highly depends on how stringent the gates are set. We set the gates for each marker such that no more than 0.2% of negative control events are located within the positive gate when recording the isotype control or unstained WT cells. The validity of these gates was then confirmed by determining the purity of the sorted cells by resorting[9], and by examining the sorted cells (by plating cells onto poly-D-lysine–coated cover slips, followed by fixation and immunocytochemistry) to ensure their correct cell identity (see Step 20).

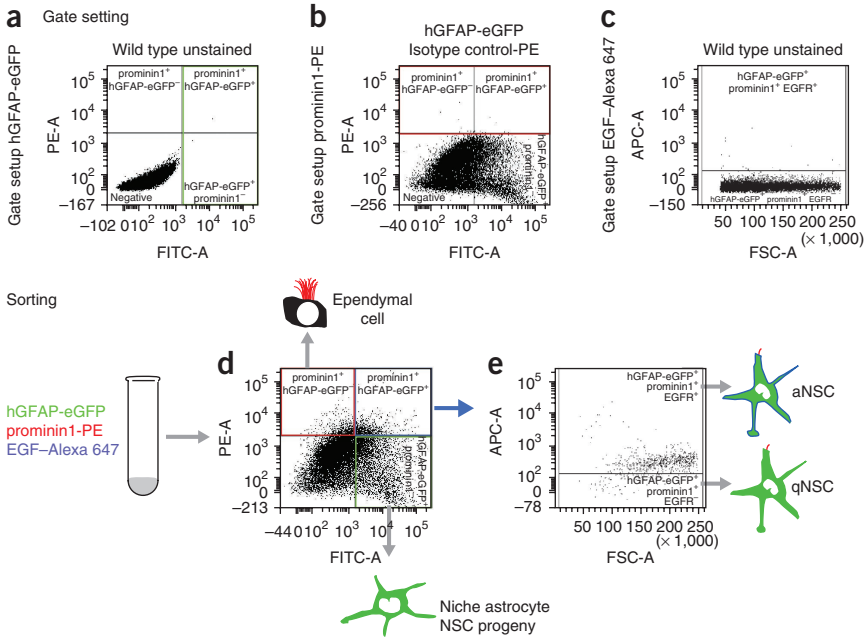

Figure 5 | Isolation of NSCs and other SEZ cells. (**a**–**c**) Gate setting for hGFAP-eGFP (**a**), prominin1 (**b**) and EGF (**c**) (see also **Fig. 4e–g**). (**d**,**e**) Purification of distinct cell types from the SEZ. (**d**) Dot plot depicting the isolation of NSCs (blue box), ependymal cells (red box) and niche astrocytes/NSC progeny (green box). Note that because of its stability, GFP could be detected in the NSC progeny[9]. (**e**) Dot plot illustrating the separation of NSCs into the EGFR$^+$ fraction (activated NSCs) and EGFR$^-$ fraction (quiescent NSCs).

! CAUTION Please be aware that the percentage of labeled cells can vary because of dissection, dissociation, filtering, staining of the samples, gate settings and FACS laser setup. The average percentage of labeled cells presented here is just a guideline.
! CAUTION Here we used a transgenic mouse line (heterozygous mice) with enhanced GFP under the hGFAP promoter[13]. Be aware that the hGFAP-eGFP$^+$–only fraction also contains some NSC progeny[9]; this fraction increases when homozygous hGFAP-eGFP animals are used.
▲ CRITICAL STEP If the proportion of dead cells is too high, do not use the samples for sorting.
? TROUBLESHOOTING

20| Examine the identity of the sorted cells by plating cells onto poly-D-lysine–coated cover slips, followed by fixation and immunocytochemistry. As *in vitro* readout for stem cell potential culture, single cells in neurosphere medium were chosen to assess their capacity of self-renewal and multipotency[9].
? TROUBLESHOOTING

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
8, 17	High proportion of dead cells	Mechanical dissociation was too harsh	Gentler mechanical dissociation
		Pasteur pipette is not fire-polished well	Improve fire-polishing of the Pasteur pipette
		PI concentration is too high	Check concentration of PI stock solution
9–11	Low number of cells	Insufficient mechanical dissociation	Enhance mechanical dissociation
		Cells stick to the Pasteur pipette or the tubes	Use FBS-coated Pasteur pipette and coat all tubes by incubating in 10% (vol/vol) FBS in PBS for a few minutes
14	No staining	Antibody is not included or is not working anymore	Repeat the staining procedure Check antibody age/storage; if necessary, order new antibody
	Low proportion of marker-expressing cells	Antibody concentration is too low	Repeat the staining procedure with increased antibody concentration (adjust concentration of the isotype control as well)
19, 20	Inefficient sorting	Concentration of cells in the sample is too high	Add more staining solution to dilute the sample
		Flow rate is too high	Reduce the flow rate to a maximum of 2,000 events per s
		FACS settings are not optimal	Optimize the FACS stream and the drop delay
		Cells are lost by adherence to sorting tubes	Use polypropylene tubes
	Insufficient purity of sorted cells	Gate settings are incorrect	Reset the gates with the correct controls
		Sorting precision mode is inadequate	Change the sorting precision mode

● TIMING
Steps 1–7, Dissection of SEZ: ~15 min per mouse
Steps 8–13, Dissociation and purification: ~1.5 h
Steps 14–17, Staining for FACS: ~50 min
Steps 18–20, FACS analysis and sorting: ~2–3 h

ANTICIPATED RESULTS

This protocol allows the isolation of self-renewing, multipotent NSCs at high purity by using a dual-labeling technique. It is also the most efficient, accurate and direct method to identify adult NSCs, as it does not require time-consuming BrdU label-retaining experiments combined with cell type–specific immunohistochemistry[26]. Furthermore, it allows the unequivocal identification of NSCs versus other cells that may become label-retaining with age or after specific treatments. Notably, any analysis using this protocol is done using primary cells directly after their isolation from the tissue, and it does not require any culturing of the cells in the presence of growth factors, which might alter the characteristics of the endogenous cells.

Following this protocol, on average, 20% of the single, living cells express hGFAP-eGFP, 9% express prominin1 and 18% are positive for EGFR. These proportions are expected to change when similar, but not identical transgenic mouse lines are used. Even by using other GFAP-GFP mouse lines[17,27], a lower or higher proportion of GFP$^+$ cells may result from different integration sites causing higher or lower gene activation, or from higher or lower fluorescence intensities due to the use of different GFP variants (GFP versus enhanced GFP, compare ref. 28 with refs. 10,29, and ref. 17 with ref. 9). For example, although all cells forming multipotent and self-renewing neurospheres were included in the GFP$^+$/prominin1$^+$ fraction when hGFAP-eGFP mice were used[9], the use of a weaker fluorescent GFP in hGFAP-GFP mice resulted in neurosphere-forming cells in the negative population[17]. Indeed, the enhanced GFP is so strong that protein levels inherited by the progeny (e.g., transit-amplifying progenitors), are still detectable by FACS. Even though neuroblasts are still included in the GFP$^+$ fraction of hGFAP-eGFP$^+$ cells[9,10], they are not included in the GFP$^+$ fraction when the hGFAP-GFP mouse line is used[17]. For the protocol described here, it is therefore important to use the mouse line described originally[13]; the mouse line should be maintained on the original background, rather than backcrossed to C57BL/6J, which results in variegation and silencing of the transgene[30].

As expected from BrdU label-retaining experiments and *in vivo* characterization, the NSCs are a small population of hGFAP-eGFP$^+$ cells that are also prominin1$^+$ (about 2.5% (ref. 9)); they can be further subdivided into the 34% of cells that are not labeled by the fluorescent EGF ligand, in contrast with 66% of the triple-positive cells. Given previous work on the correlation of EGF receptor labeling with activation of NSCs, these populations should contain the quiescent and activated NSCs[17,23]. Moreover, this protocol can be extended to select neuroblasts and transit-amplifying progenitors[17], thereby allowing isolation of the entire SEZ lineage. This is very useful for quantitatively determining alterations in the adult NSC and neurogenic lineage in mouse mutants, pharmacological treatments, diverse injury conditions and aging.

Prospective isolation of NSCs in these conditions allows not only for accurate quantification of their numbers, but also for the assessment of changes in transcription of NSCs, either on a genome-wide basis by microarray or deep sequencing methods or by assessing the expression of specific candidate genes. Thus, potential changes in gene expression of NSCs in mutants, particularly after treatment or in disease conditions, can then be compared with the transcriptome as previously demonstrated[9], with the aim of identifying alterations in pathways that regulate NSC behavior.

The protocol also provides the possibility of studying the mechanisms of progression of NSCs from a more quiescent (EGFR-negative) to a more activated (EGFR-positive) state. This allows the identification of factors that can be used to activate the progression within the lineage and that might have a role in the endogenous stem cells' capacity to react to brain injuries. Moreover, it enables the possibility of identifying a new surface marker for the delineation of the NSCs, as a limitation of this protocol is the necessity of using transgenic hGFAP-eGFP mice.

Beyond expression analysis, sorted cells can be cultured either as neurospheres or in adherent culture without any growth factors, which allows the assessment of the further lineage progression and the cell division mode by continuous live imaging[23,24]. Sorted NSCs can then also be cocultured on distinct matrix substrates or in combination with distinct cell types from the niche (neuroblasts, transient amplifying progenitor cells, ependymal cells, niche astrocytes) to dissect key influences from the stem cell niche by examining the influence of distinct niche components on the behavior of NSCs. Taken together, this protocol finally allows the assessment of the intrinsic and extrinsic cellular and molecular mechanisms regulating NSC behavior.

Note: Supplementary information is available via the HTML version of this article.

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