

Video Article

Isolation and Culture of Adult Neural Stem Cells from the Mouse Subcallosal Zone

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Abstract

Adult neural stem cells (aNSCs) can be used for the regeneration of damaged brain tissue. NSCs have the potential for differentiation and proliferation into three types of cells: neurons, astrocytes, and oligodendrocytes. Identifying aNSC-derived regions and characterizing the aNSC properties are critical for the potential use of aNSCs and for the elucidation of their role in neural regeneration. The subcallosal zone (SCZ), located between white matter and the hippocampus, has recently been reported to contain aNSCs and continuously give rise to neuroblasts. A low percentage of aNSCs from the SCZ is differentiated into neurons; most cells are differentiated into glial cells, such as oligodendrocytes and astrocytes. These cells are suggested to have a therapeutic potential for traumatic cortical injury. This protocol describes in detail the process to generate SCZ-aNSCs from an adult mouse brain. A brain matrix with intervals of 1 mm is used to obtain the SCZ-containing coronal slices and to precisely dissect the SCZ from the whole brain. The SCZ sections are initially subjected to a neurosphere culture. A well-developed culture system allows for the verification of their characteristics and can increase research on NSCs. A neurosphere culture system provides a useful tool for determining proliferation and collecting the genuine NSCs. A monolayer culture is also an *in vitro* system to assay proliferation and differentiation. Significantly, this culture system provides a more homogenous environment for NSCs than the neurosphere culture system. Thus, using a discrete brain region, these culture systems will be helpful for expanding our knowledge about aNSCs and their applications for therapeutic uses.

Video Link

The video component of this article can be found at <https://www.jove.com/video/54929/>

Introduction

NSCs have characteristics of self-renewal and multiple-lineage differentiation. To confirm these properties, a neurosphere culture system has widely been used. The neurosphere culture system was developed in the early 1990s and served as a standard stem cell culture system¹. Depending on self-renewal potency, NSCs continuously proliferate and generate a cell mass in a suspension culture. The number of cells and the size of the neurosphere are considered to be closely associated with the proliferation properties of the NSCs. Monolayer cultures are also widely used for the maintenance and differentiation of NSCs. Compared to the neurosphere culture, the monolayer culture system provides better homogenous maintenance and expansion of NSCs². These two well-developed culture systems have contributed to the characterization of aNSCs *in vitro*.

NSCs reside in different brain regions, such as the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampus³⁻⁵. The subcallosal zone (SCZ) of the caudal subcortical white matter is recognized as a novel neurogenic region⁶⁻⁸. It was recently reported that the SCZ-aNSCs have therapeutic potential in traumatic brain injury⁹. Compared to other neurogenic regions, the SCZ resides along the subcortical white matter. In the human brain, subcortical white matter occupies a larger region than in the mouse brain¹⁰. Therefore, an understanding of the characteristics of SCZ-aNSCs using an *in vitro* culture system is important in order to promote the potential use of these cells for neural regeneration. Precise dissection of the desired brain region is required to rule out possible contamination by unwanted regions containing active or quiescent NSCs. For instance, aNSCs in non-neurogenic regions can be activated and produce new neural cells in injured brains or during *in vitro* culturing¹¹. To obtain NSCs from the SCZ, cells were collected from brain slices containing the SCZ. Then, a careful micro-dissection of the SCZ region was performed using a fine needle. To generate the neurosphere from the SCZ, micro-dissected SCZ tissue chunks were dissociated into single cells and then cultured as a suspension in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). After SCZ-aNSCs form neurospheres, they also can be maintained as neurospheres or monolayers for expansion. This procedure also demonstrates immunostaining processes with various markers for the detection of NSCs and their progeny after their expansion and differentiation in a monolayer culture. Here, a visual protocol of the SCZ-aNSC culture system is presented. This protocol contains detailed instructions for the micro-dissection of the SCZ region and for the maintenance and passaging of the cells.

Protocol

1. Preparation of Materials and Culture Medium

- For the dissection and dissociation of the SCZ, wrap the brain matrix, double-edged razor blade, and forceps with aluminum foil, and then sterilize them by autoclaving.
- Prepare 50 ml of cold PBS buffer to wash the whole mouse brain.
- Set up a dissection microscope and prepare the surgical tools required for the dissection of the brain (autoclaved scissors and forceps) and the isolation of the SCZ (1 ml syringe, 30 G needle, brain matrix, and fine forceps).
- Preparation of N2 Medium:
 - Prepare F-12/DMEM (+L-glutamin, +sodium bicarbonate) medium with 2% B27, 1% N2 supplements, and 1% Penicillin-Streptomycin. Note: The growth medium consists of N2 medium and growth factors (20 ng/mL purified epidermal growth factor (EGF) and 20 ng/mL basic fibroblast growth factor (bFGF2)).
- Prepare a digestion buffer (40 unit/ml papain, 2.4 unit/ml dispase II, and 2% penicillin-streptomycin in PBS) for tissue digestion.
- Preparation of Coating Plate and Coverslip:
 - Prepare poly-L-ornithine (PLO; 0.01%) and laminin (10 µg/ml dissolved in DH₂O). To coat 6-well plates for the maintenance of SCZ-aNSCs as a monolayer or 18 mm coverslips for immunostaining, incubate them with PLO overnight at 4 °C. Then wash them 3 times with DH₂O. Allow the plates and coverslips to dry after the last washing.
 - Next, incubate the plates with laminin overnight at 4 °C. Then, wash them 3 times with DH₂O. Caution: Do not dry the laminin, which will affect cell attachment. Note: The coating solutions can be reused 3 times.

2. Isolation and Dissociation of the Adult SCZ

- Prior to culture preparation, place the brain matrix and double-edged razor on ice. Note: Do not freeze the brain matrix, because the brain could attach to it.
- Sacrifice a mouse (8 weeks old) by CO₂ asphyxiation or cervical dislocation.
 - Cut off the head with sharp scissors after spraying 70% ethanol. To immobilize the head, hold both sides of the head tightly. Cut the skin with scissors at the midline in a caudal-rostral direction. This promotes the complete removal of the skin from the skull.
 - Remove the caudal part of skull (posterior to lambda) first, and then insert the forceps between the skull and brain at the dorsal midline position. Grab the left (or right) parietal bone and carefully peel it off. Repeat this procedure for the removal of the other parietal bone. Note: Disconnection of optic nerve and removal of the meninges from the brain make it easier to detach the brain from the skull.
 - Transfer the brain to cold PBS buffer (25 ml) and rinse it twice to remove excess blood.
- Place the brain into the brain matrix on ice and make coronal cuts to obtain 1 mm thick slices. Transfer the brain slices (1 mm) containing the SCZ regions that are located in the posterior part of the brain to a cold PBS in a 35 mm plastic petri dish. Caution: In order to obtain a parallel plane of coronal sections, ensure that the brain fissure is placed in the midline of the brain matrix; it is critical to avoid unnecessary variations in the sections. Note: Obtain brain slices at 2-3 mm posterior to bregma; this allows the separation of the SCZ from the SVZ.
- Under the dissecting microscope with a low magnification, micro-dissect the SCZ from the white matter area of the cortex and hippocampus with a bent 30G needle^{6,9}. Then, remove the cortex regions above the SCZ. Place the dissected SCZ region from the slices into a 35 mm plastic petri dish on ice without cold PBS. Note: Contamination of extra regions containing mature neurons could affect the viability of aNSCs and neurosphere formation because they undergo cell death in aNSC culture conditions.
- Using a bent 30 G needle, immediately chop the dissected tissues into small pieces. Note: If a longer time is required for dissecting the SCZ tissue, immerse the dissected tissues in cold PBS before chopping.
- Re-suspend the chopped tissue with 1 ml of digestion buffer, transfer the tissue to a 15 ml tube containing 2 ml of digestion buffer, and incubate it for 30 min in a 37 °C water bath. Note: Shake the tube every 10 min to mix well.

3. Subcallosal Zone-derived Adult Neural Stem Cell Culture

- Tap the tube mildly to dissociate the digested tissue, and then centrifuge the tube at 145 x g for 5 min. Discard the supernatant, re-suspended the digested tissue with 1 mL of pre-warmed N2 medium to wash out the digestion buffer, and gently pipet the sample solution a maximum of 5 times using a P1000 pipette. Note: Over-triturating with a narrow pipet tip can diminish cell viability and subsequent growth.
- Centrifuge the tube at 145 x g for 5 min. After discarding the supernatant, re-suspend the cell pellet in 1 ml of N2 medium.
- Prepare 1 ml of N2 medium in a non-coated 6-well plate and add 1 ml of the suspended cells to make a final volume of 2 ml.
- Add EGF (20 ng/ml) and bFGF (20 ng/ml) into each well. Gently shake the 6-well culture dish by hand to mix the added growth factors with the plated cells. Keep the 6-well plate in a 37 °C and 5% CO₂ incubator.
- Add EGF (20 ng/ml) and bFGF (20 ng/ml) to each well every day for 8 days. Every third day, add 200 µl of N2 media to maintain the approximate 2 ml volume of the medium.

4. Passaging of NSCs as Neurospheres and to Monolayer Cultures

1. Gather the neurospheres and transfer them to a new 15 ml conical tube.
Note: The number of neurospheres (>50 μm diameter) per well from the SCZ of a single mouse brain was 64.3 ± 7.31 , which was less than that of the SVZ (190.5 ± 6.33)⁹.
2. Incubate the neurospheres with 0.5 ml of dissociation buffer for 5 min in a 37 °C water bath to dissociate the neurospheres into single cells. Primary neurospheres can be dissociated into single cells and maintained over several passages as neurosphere or monolayer cultures.
Note: Expanding SCZ-aNSCs as a monolayer is superior to neurospheres because SCZ-aNSCs can be passaged >10 times in a monolayer culture format, but <5 times in a neurosphere culture format.
Caution: SCZ-aNSCs exhibit strong aggregation, and it is difficult to dissociate them into single cells. Thus, the neurosphere culture format is not recommended for routine cell expansion.
3. Gently pipet the sample solution up and down with a P1000 pipette less than 5 times and centrifuge the tube at 145 x g for 5 min. Discard the supernatant and re-suspend the neurospheres with 1 ml of N2 medium.
Note: Over triturating with a narrow pipette tip can diminish cell viability and subsequent growth.
4. To count the cells, make a 1:1 mixture of the cell suspension (10 μl) and the 0.4% trypan blue solution, and then count the number of live cells on a hemacytometer. After coating a 6-well plate with PLO/laminin, plate the cells at 2.5×10^5 cells/ml with 2 ml of N2 medium for each well.
Note: Changes in cell density can affect their condition and differentiation potential.
5. Maintain the SCZ-aNSCs with a daily treatment of growth factors (2 ml, 20 ng/ml) for 5 days, and then passage them.

5. Differentiation of Subcallosal Zone-derived Adult Neural Stem Cells

1. Plate aNSCs onto a PLO/Laminin-coated 18 mm coverslip with 1×10^5 cells/ml in 1 ml N2 with growth factors (20 ng/ml) for differentiation of the SCZ-aNSCs.
2. The next day, when the cells are firmly attached to the coverslip, exchange the growth medium with N2 to remove the growth factors.
3. After 6 days, wash the differentiated cells with 1 ml of PBS to remove cell debris and fix them for immunostaining.
Note: BrdU can be incorporated into the newly-synthesized DNA of proliferating cells. Therefore, if desired, BrdU (10 $\mu\text{g/ml}$) can be added to live cells before the fixation of cells.

6. Immunostaining Adult Neural Stem Cells and Differentiated Progenitors

1. For immunostaining, wash the cells with PBS and fix them with 4% PFA for 20 min at room temperature.
Caution: PFA is highly toxic; avoid contact with the skin and eyes.
2. Remove the 4% PFA, rinse the fixed cells with PBS 3 times, and then store them at 4 °C until they are needed for immunostaining.
3. Incubate the cells on the coverslip with blocking solution (3% bovine serum albumin and 0.1% Triton X-100 in 1x PBS) for at least 30 min at room temperature.
4. Prepare the primary antibodies in fresh blocking solution and incubate the samples overnight at 4 °C.
 1. Immunostaining the aNSCs
 1. Stain the aNSCs using anti-Nestin and anti-BrdU antibodies. Before fixation, add 20 μg of BrdU to the aNSCs and incubate them for 2 h. Perform the denaturing step using 2 N HCl for 20 min at 37 °C before performing the blocking step.
 2. Immunostaining the differentiated progenitors:
 1. Use anti-O4, anti- β -tubulin, and anti-glial fibrillary acidic protein (GFAP) antibodies to label the differentiated progenitors.
5. Wash the samples with PBS 3 times and incubate them with secondary antibodies conjugated to fluorescent dyes (1:500) in blocking solution for 30 min at room temperature. Then, wash the samples 3 times with PBS.
Note: Use secondary antibodies that match the hosts of the primary antibodies. Hoechst33343 (1:2,000) is used for nuclear staining.
6. Add mounting solution to a slide glass and proceed with mounting. Observe and image the sample on a confocal microscope at multiple wavelengths: FITC (488 nm), Cy3 (543 nm), Cy5 (647 nm), and Hoechst33343 (405 nm).

Representative Results

Defining the culture system for aNSCs from the unknown neurogenic region is essential for understanding these cells and for developing their potential use in brain repair¹². It is known that NSCs in different developmental stages or in different regions behave differently^{3,4}. Recently, it was reported that SCZ-derived cells exhibit differential potentials for neuronal differentiation *in vivo* and *in vitro* compared to SVZ-derived cells⁷⁻⁹. Therefore, to precisely isolate each neurogenic region, brain slices that include the SCZ were dissected using a 1 mm brain matrix (**Figure 1A**). After 8 days of culture with growth factors, aNSCs derived from the SCZ can form neurospheres, in which the cells can be subsequently maintained (**Figure 1B**).

Since a subset of NSCs in the neurospheres may be spontaneously differentiated¹³, a monolayer culture system is also helpful for the maintenance of the relatively homogeneous population of SCZ-aNSCs. Growth factors and dissociation enzymes such as trypsin do not easily permeate deep inside of the neurosphere^{14,15}. Monolayer cultures provide more even conditions for the expansion of NSCs. From primary neurospheres, SCZ-aNSCs were dissociated into single cells by a digestion buffer treatment. One day after cell seeding, aNSCs were attached to the coated plate and exhibited cell proliferation (**Figure 2A**). To confirm their potency of proliferation, BrdU was added into the media. After incubation with BrdU for 2 hr, cells were readily stained with anti-BrdU (a marker for proliferation) and anti-Nestin (a marker for neural stem cells) antibodies, indicating that SCZ-aNSCs are actively proliferating and maintaining the key properties of stem cells (**Figure 2B**). Accordingly, they did not exhibit markers for differentiated cells, such as EGFR (expressed in transiently-amplifying cells) and DCX (expressed in neuroblasts) (**Figure 2C**).

To confirm the multiple differentiation potential of SCZ-aNSCs, growth factors were removed from the culture media. After 6 days, cells were immunostained with various makers for differentiated cells. To exhibit the different progenies of aNSCs, markers for neurons (Tuj1), astrocytes (GFAP), and oligodendrocytes (O4) were employed; all of these cell types were generated from the SCZ-aNSCs (**Figure 3**).

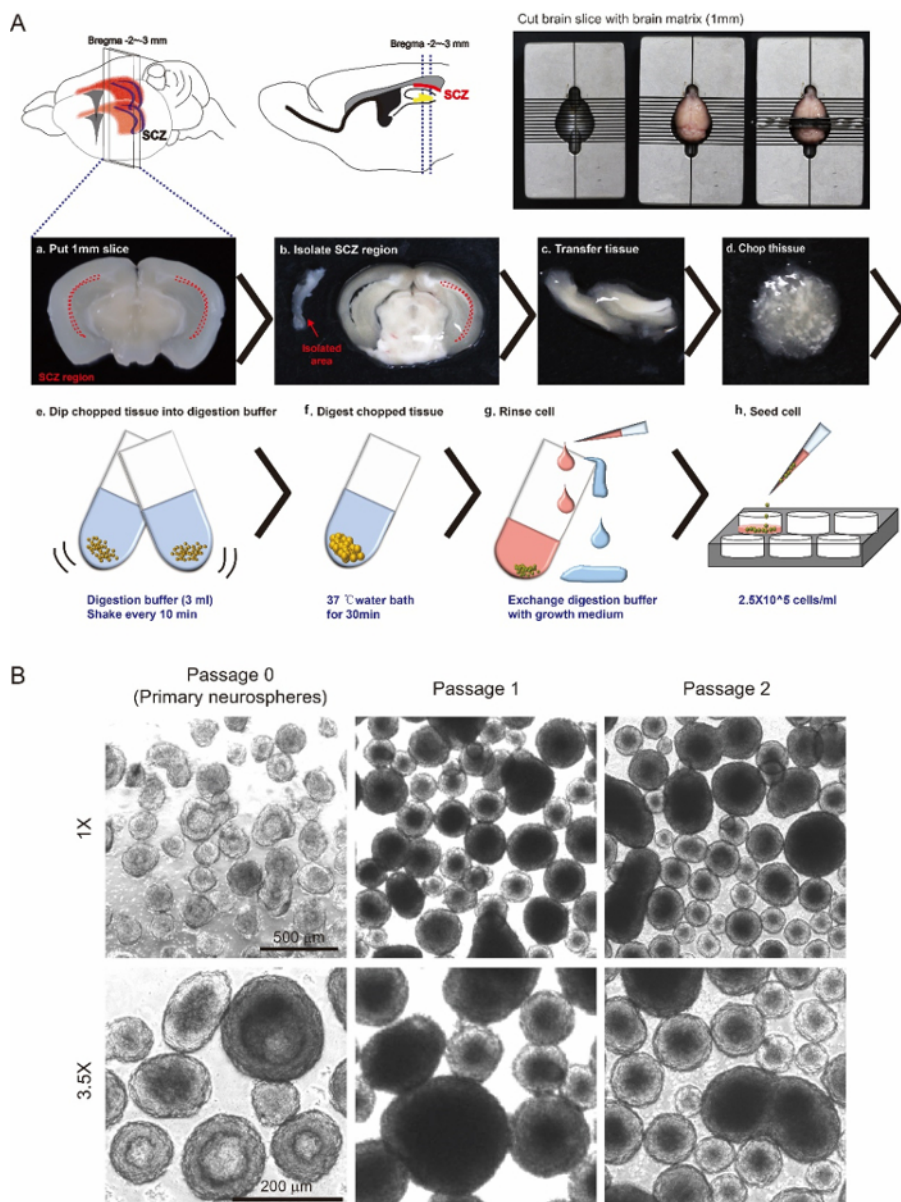


Figure 1: Isolation of the SCZ region and formation of the neurosphere. (A) Procedure for the dissection of the SCZ region from the adult mouse brain. To culture the SCZ-aNSCs, an 8 week-old mouse brain is placed onto a brain matrix (1 mm intervals). After sectioning, 1 mm brain slices that included the SCZ region (2-3 mm from bregma) were dissected (indicated by the red dotted line). (B) Neurosphere formation. Eight days after the *in vitro* culture, primary neurospheres (passage 0) were formed and passaged. [Please click here to view a larger version of this figure.](#)

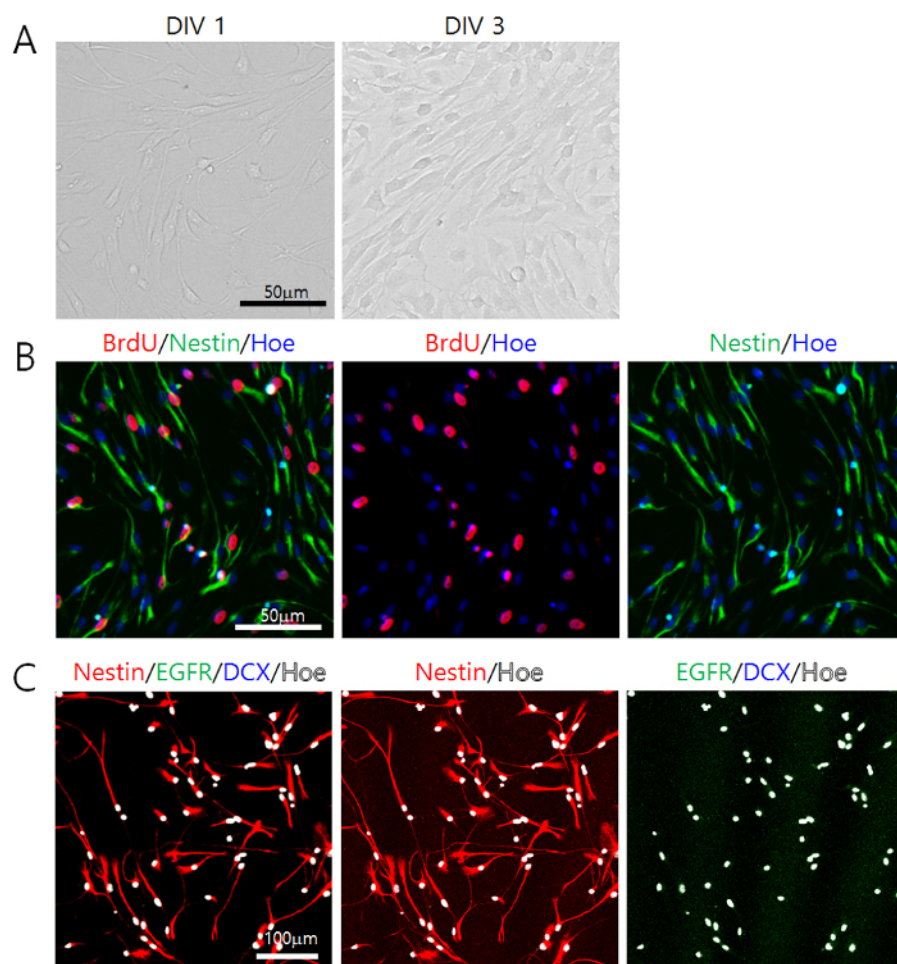


Figure 2: Maintenance of the SCZ-aNSCs as a monolayer culture. (A) One day after seeding the dissected cells, SCZ-aNSCs were attached and expanded onto a coated dish in a monolayer manner (left). Three days after maintenance, the number of SCZ-aNSCs was increased (right). (B) Immunostaining with BrdU (red, a marker for proliferating cells), Nestin (green, a marker for neural stem cells), and Hoechst33343 (blue, a marker for nuclei). (C) Immunostaining with neural stem/progenitor cell markers Nestin (red, a marker for type B neural stem cells), EGFR (green, a marker for type C transient-amplifying cells), and DCX (blue, a marker for type A neuroblasts). Nuclei were counterstained with Hoechst33343 (white). [Please click here to view a larger version of this figure.](#)

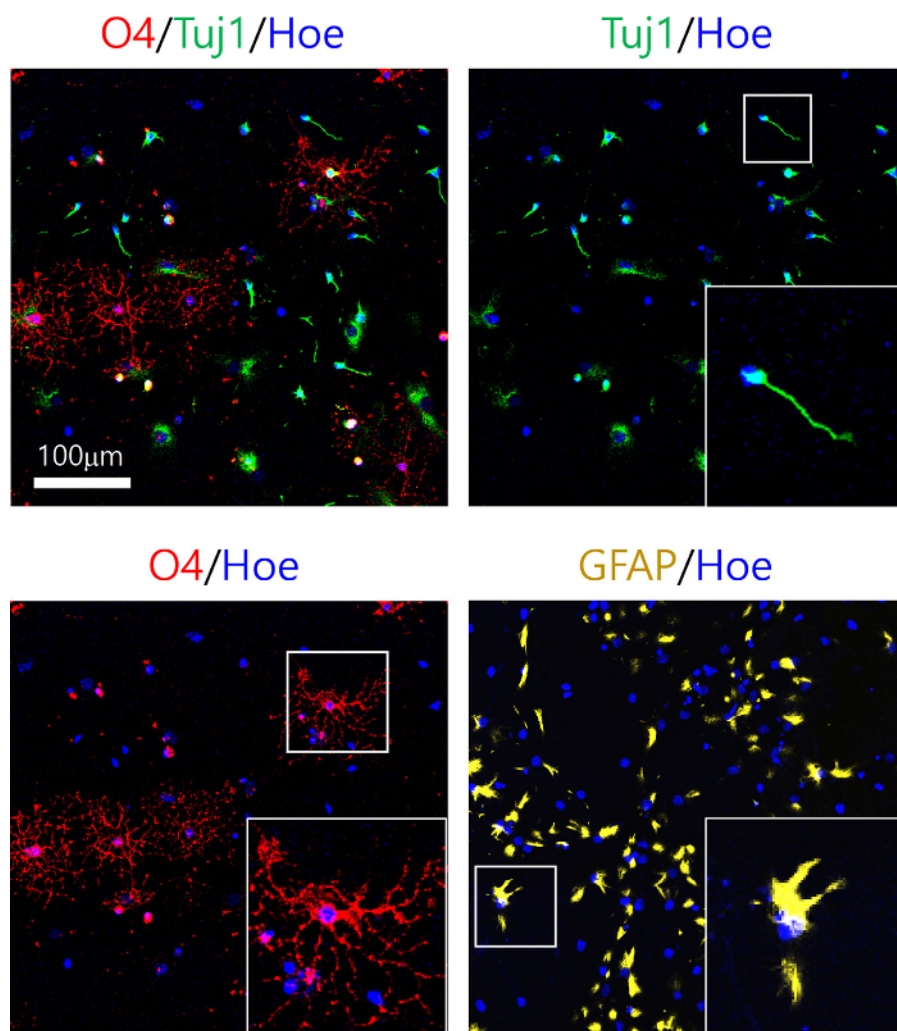


Figure 3: Immunostaining of the differentiated cells from the SCZ-aNSCs. Immunostaining with differentiation markers Tuj1 (green, a marker for immature neurons), O4 (red, a marker for oligodendrocytes), and GFAP (yellow, a marker for astrocytes). Magnified images are shown as insets. Hoechst33343 (blue) was used for counter-staining the nuclei. [Please click here to view a larger version of this figure.](#)

Discussion

This paper describes a detailed protocol to generate NSCs from the adult mouse SCZ and to maintain them for various applications. There are three critical steps for establishing the *in vitro* culture system needed to purify and expand SCZ-NSCs. First, it is important to ensure that the SCZ region is precisely dissected out from other potential neurogenic regions (**Figure 1B**). Thick and precise sections containing the SCZ regions were obtained with a 1 mm interval brain matrix, and then a fine needle was used for the micro-dissection of the SCZ from other cortical regions (**Figure 1A**). When non-NSCs from adjacent tissues, such as the cerebral cortex, are cultured with SCZ-aNSCs, catastrophic death occurs, which negatively affects the viability and sphere-formation of SCZ-NSCs^{16-18,22}. The caudal SCZ (2-3 mm posterior to bregma) was confirmed as the best region for generating distinct SCZ-aNSCs. Second, the appropriate enzymatic treatment in the harvesting and passaging steps is critical for achieving a high yield of cells. Dispase II and papain were more effective for isolating aNSCs than trypsin. Dissociation of cells for passaging with dissociation buffer instead of trypsin enhanced their viability¹⁹. Mechanical dissociation and trituration with a pipette should be minimal. Third, a cell strainer is generally used in primary culture systems to remove cellular debris after tissue digestion. However, due to the localization of SCZ-aNSCs, these cells are obtained after the breakage of white matter by enzyme digestion and mechanical trituration. During filtration with a cell strainer, a substantial amount of cells would be lost. Therefore, culturing SCZ-aNSCs without using a cell strainer is a better way to get a high yield of cells.

While both neurosphere cultures and monolayer cultures can be applied to the maintenance of NSCs, one limitation of the neurosphere culture is that single NSCs dissociated after splitting can be randomly aggregated. The aggregation results in different sizes of neurospheres. When the size of a neurosphere reaches a certain critical value, the neurosphere grows as a heterogeneous structure, due to a lack of nutrients, growth factors, and oxygen at the core²⁰. Furthermore, neurospheres with large sizes are not easily dissociated with dissociation buffer and require longer enzymatic treatment times with extensive mechanical trituration, leading to lower cell viability. Therefore, the monolayer culture system is recommended to maintain SCZ-aNSCs through multiple passages. In a monolayer culture system, aNSCs are stably maintained as NSCs (type B) without spontaneous differentiation into specified cells, such as progenitors (types C and A) (**Figure 3A**). SCZ-aNSCs were passaged for extended periods, <5 passages in a neurosphere format and >10 passages as a monolayer. This is consistent with previous results, which suggest that the monolayer culture system maintains NSCs *in vitro* in long-term cultures²¹. However, the proliferating speed

decreased, and the portion of dying cells increased over 5 passages. Extended passaging affects the multipotency and neuronal differentiation with increased chromosome aberration²². Therefore, to avoid extended passaging effects, it is recommended to use early passage (<passage 5) cells for proliferation and differentiation analysis. Although the potential for self-renewal of SCZ-aNSCs is similar to SVZ-aNSCs, less neuronal differentiation was shown in SCZ-aNSCs⁹.

Methods for isolating aNSCs from neurogenic regions of the adult brain, including the SVZ and dentate gyrus (DG), have been established²². Although such protocols have promoted the isolation and cultivation of aNSCs *in vitro*, there are several limitations to obtaining a high number of cells. Many protocols utilize a brain tissue chopper that may cause the loss of brain tissue during the chopping procedure. Another approach to isolate aNSCs from neurogenic regions uses a coronal cut through the brain using a scalpel. This is followed by the micro-dissection of the SVZ or of the DG along the longitudinal fissure²³. The presence of other brain regions can cause other cell types to contaminate the aNSC culture, which might affect the viability of the cells *in vitro*. With the current protocol, many different samples can be managed in a single experiment, including controls versus a variety of experimental groups. Also, slicing using a brain matrix is superior to a brain chopping tool, as it allows for the attainment of NSCs from various brain regions with a high cell yield. It also enables the comparison of aNSCs from different regions of the same brain.

Transplantation and engineering of endogenous NSCs have been considered as possible strategies for stem cell therapy. For this, *in vitro* studies about the characteristics of aNSCs should also be comprehensively explored. Therefore, the establishment of a well-characterized *in vitro* culture system will be helpful for furthering the application of NSCs. In this culture system, the stem-cell properties of SCZ-aNSCs were well-maintained, as evidenced by self-renewal and multiple-lineage differentiation under the appropriate conditions. Therefore, this culture system can be used for the expansion of SCZ-aNSCs for biological studies and therapeutic applications.

Disclosures

The authors declare no conflicts of interest.

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