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Isolation and Expansion of Neurospheres from Postnatal (P1−3) Mouse Neurogenic Niches

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TITLE:

Isolation and Expansion of Neurospheres from Postnatal (P1–3) Mouse Neurogenic Niches

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SUMMARY:

In this article, we describe, in detail, a protocol for the generation of neurosphere cultures from postnatal mouse neural stem cells derived from the main mouse neurogenic niches. Neurospheres are used to identify neural stem cells from brain tissue allowing the estimation of precursor cell numbers. Moreover, these 3D structures can be plated in differentiative conditions, giving rise to neurons, oligodendrocytes and astrocytes, allowing the study of cell fate.

ABSTRACT:

The neurosphere assay is an extremely useful in vitro technique for studying the inherent properties of neural stem/progenitor cells (NSPCs) including proliferation, self-renewal and multipotency. In the postnatal and adult brain, NSPCs are mainly present in two neurogenic niches: the subventricular zone (SVZ) lining the lateral ventricles and the subgranular zone of the hippocampal dentate gyrus (DG). The isolation of the neurogenic niches from postnatal brain allows obtaining a higher amount of NSPCs in culture with a consequent advantage of higher

yields. The close contact between cells within each neurosphere creates a microenvironment that may resemble neurogenic niches. Here, we describe, in detail, how to generate SVZ- and DG-derived neurosphere cultures from 1−3-day-old (P1−3) mice, as well as passaging, for neurosphere expansion. This is an advantageous approach since the neurosphere assay allows a fast generation of NSPC clones (6−12 days) and contributes to a significant reduction in the number of animal usage. By plating neurospheres in differentiative conditions, we can obtain a pseudomonolayer of cells composed of NSPCs and differentiated cells of different neural lineages (neurons, astrocytes and oligodendrocytes) allowing the study of the actions of intrinsic or extrinsic factors on NSPC proliferation, differentiation, cell survival and neuritogenesis.

INTRODUCTION:

The neurosphere assay (NSA) was firstly described in 1992[1,2] and still remains a unique and powerful tool in neural stem cell (NSC) research. The isolation of NSCs from the main neurogenic regions has challenging issues because the requirements to maintain these cells in physiological conditions remain poorly understood. In the NSA, cells are cultured in a chemically defined serum-free medium with the presence of growth factors including the epidermal growth factor (EGF) and the basic fibroblast growth factor (bFGF)[1–3]. Neural precursor cells (stem and progenitors) are selected by using these mitogens since these cells are EGF and FGF-responsive entering a period of active proliferation while other cells, namely differentiated cells, die[4]. Neural precursor cells grow as neurospheres, which can be then passaged to further expand the pool of these cells[5]. Importantly, since these neural stem progenitor cells (NSPCs) are multipotent they are able to differentiate into the three major cell types of the central nervous system (CNS): neurons, oligodendrocytes and astrocytes[5].

The NSA provides a renewable source of undifferentiated CNS precursors, which can be used to study several processes including NSC proliferation and self-renewal, and neuronal and glial differentiation, in both physiologic and disease context. Moreover, in vitro studies can be used to evaluate the degree of intrinsic specification present in neural precursors during development, as well as to study the full potential of the cells, by removing extrinsic cues associated with their normal environment[6]. The neurosphere model is valuable to evaluate putative regulators since by maintaining the cells in a medium devoid of serum, the environmental cues are only provided by the surrounding cells[6]. Moreover, in the NSA, NSPCs are easily expanded in culture, the density of cells per area is high and the heterogeneous composition of the neurospheres has some similarity to in vivo niches[6]. These well-established advantages are the reason why this methodology has been widely used by many researchers.

The following protocol describes in detail all the processes from the isolation of postnatal NSPC population from the two main neurogenic regions, the subventricular zone (SVZ) and the hippocampal dentate gyrus (DG), to the expansion of those cells as neurospheres, as well as to the differentiation into neurons, astrocytes and oligodendrocytes. Lastly, different assays are also described to access stemness and multipotency properties of SVZ- and DG-derived NSPCs.

PROTOCOL:

All experiments were performed in accordance with the European Community (86/609/EEC; 2010/63/EU; 2012/707/EU) and Portuguese (DL 113/2013) legislation for the protection of animals used for scientific purposes. The protocol was approved by the "iMM's institutional Animal Welfare Body – ORBEA-iMM and the National competent authority – DGAV (Direcção Geral de Alimentação e Veterinária)."

1. Basic setup and preparation of culture medium

1.1. On the day of dissection, prepare the appropriate amount of growth medium corresponding to serum-free medium (SFM) composed of Dulbecco's modified eagle medium [(DMEM)/F12 with L-glutamine] (**Table of Materials**) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin (pen/strep), 1% B27, with also 10 ng/mL EGF and 5 ng/mL bFGF. Warm the culture medium to 37 °C in a water bath.

NOTE: The volume of growth medium depends on the number of pups, for 5 pups prepare ~100 mL (50 mL for SVZ and 50 mL for DG); however, after counting the number of cells (step 5.1) the exact volume will have to be adjusted.

1.2. For microdissection of SVZ and DG, prepare the calcium and magnesium-free Hanks' balanced saline solution (HBSS) dissection medium supplemented with 100 U/mL pen/strep.

NOTE: Prepare 50−100 mL of dissection medium.

1.3. Set up a dissection microscope and prepare the tools needed to remove the brain (scissors and small spatula) and for SVZ and DG microdissections (Dumont small scissors, #7 forceps, #5 forceps, #5S forceps) by soaking in 70% ethanol.

2. Harvesting of postnatal (P1–3) mouse brains and SVZ/DG microdissections

2.1. Prepare 60 mm Petri dishes (growth area 21 cm^2) with HBSS supplemented with pen/strep and 2 sample tubes (one for SVZ and one for DG) with 500 µL of supplemented HBSS each.

2.2. Euthanize mice pups (P1−3) according to the protocol approved by the Institutional Animal Care facility/guidelines. Perform decapitation with a single incision with sharp scissors at the base of the brainstem.

2.3. Holding the ventral part of the body at the base of the head and using small pointed scissors, make a midline incision in the skin over the entire length of the head, thus revealing the surface of the skull.

2.4. Make a longitudinal incision at the base of the skull and continue cutting along the sagittal suture using small scissors with an angle shallow as possible in order to avoid damaging the brain structures.

2.5. Peel the skull to the sides using curved forceps and expose the brain.

CAUTION: Make sure the dissecting instruments are free of ethanol before touching the brain.

2.6. Isolate the brain from the skull using a small spatula, by sliding under the base of the brain to cut the cranial nerves and blood vessels that are connected to the base of the brain, and transfer the brain into a Petri dish containing cold supplemented HBSS solution.

2.7. Place the Petri dish containing the brain under a dissecting microscope at low magnification and position the brain on its dorsal surface.

2.8. Using fine forceps, remove the meninges from the ventral side of the brain and the olfactory bulbs, while holding the brain in position by the cerebellum. Rotate the brain onto the ventral aspect and peel off the rest of the meninges.

NOTE: Removing the dorsal meninges is a crucial step to ensure correct brain slicing.

2.9. Discard the cerebellum making a cut using forceps. Place a filter paper with a pore size of 11 µm onto a tissue chopper (**Table of Materials**) and set the brain onto the filter paper using curved-pointed forceps. Chop the brain into 450 µm coronal sections and use a wet lamina to collect the sectioned brain into a new Petri dish filled with cold supplemented HBSS.

2.10. To dissect out the SVZ, use forceps to separate coronal slices in an anterior-to-posterior fashion until reaching slices with the lateral ventricles, under a dissecting microscope.

2.11. Cut the thin layer of tissue surrounding the lateral wall of the ventricles (which corresponds to the SVZ) with fine forceps, excluding the striatal parenchyma and the corpus callosum. Isolate the SVZ by placing the tip of the forceps in the lateral corners of the lateral ventricle: one immediately under the corpus callosum and the other into the tissue immediately adjacent to the ventral area of the lateral ventricle. Then, cut a small line of tissue surrounding the lateral ventricle.

2.12. Collect the dissected tissue into a sample tube with supplemented HBSS solution previously identified as SVZ.

NOTE: Exclude the SVZ in slices where both the lateral ventricles and the hippocampal formation begin to appear.

2.13. Go through all slices after SVZ microdissection in an anterior-to-posterior fashion and reach the hippocampal formation. Using forceps discard the first slice with hippocampus where DG is still unrecognizable.

2.14. To remove the DG, first isolate the hippocampus from the slices. Refocus the microscope to visualize the borders around DG.

2.15. Dissect the DG portion by performing a cut between the DG and CA1 region followed by a vertical cut between the DG and CA3 region using forceps. Remove fimbria and any adjacent tissue.

NOTE: In P1−3 animals, the DG is almost undistinguishable from Ammon's horn but displays a small tip.

2.16. Collect the dissected tissue into a sample tube containing supplemented HBSS solutions previously identified as DG.

NOTE: Overall injury to the hippocampus or surrounding area will make it more difficult to isolate the DG. Using an atlas of the postnatal mouse brain is essential when the user is not familiarized with the isolation of the DG and SVZ tissue from coronal sections.

3. Tissue dissociation

3.1. To dissociate the SVZ and DG tissue present in their respective tubes, add trypsin-EDTA 0.05% to have a final concentration of 5−10% of Trypsin-EDTA 0.05% in HBSS. Incubate for approximately 15 min at 37 °C, until the tissue is clumped together.

3.2. Wash the tissue from the trypsin by removing the media and adding 1 mL of new HBSS supplemented solution for 4 consecutive times.

3.3. Remove the HBSS and resuspend the digested tissue in 1 mL of SFM supplemented with 10 ng/mL EGF and 5 ng/mL bFGF. Mechanically dissociate the pellet by gently pipetting up and down approximately 7−10x using a P1000 pipette, until getting a homogenous cell solution.

CAUTION: Excessive mechanical dissociation can lead to increased cell death and will negatively impact subsequent cell growth.

4. Cell-pair assay to study cell fate

4.1. Prior to the experiment, prepare poly-D-lysine (PDL) coated 24-well plates for adherent monolayer cultures according to section 8.

4.2. To count the number of SVZ or DG cells (obtained in section 3) to be plated, use a solution containing 0.2% Trypan blue and count the cells using a hematocytometer.

4.3. Dilute the dissociated cell suspension in SFM supplemented with 5 ng/mL EGF and 2.5 ng/mL bFGF (low EGF/bFGF) at a density of 11,300 cells/cm^2 and plate them on PDL coated glass coverslips.

4.4. After 24 h, fix the cells for immunocytochemistry against NSC markers such as sex

determining region Y-box 2 (Sox2) and nestin as well as with a marker of the neuronal lineage (namely doublecortin [DCX], for immature neurons) (see section 14).

NOTE: Sox2 is a marker of NSCs that undergo mitosis. $Sox2^{+/+}$ cell-pairs resulting from a single progenitor cell division reflects stem cell expansion[7].

5. Expansion of postnatal neural stem cells as neurospheres

5.1. To determine the density of the dissociated DG or SVZ cell suspension (obtained in section 3), count the cells using a hematocytometer.

5.2. Dilute SVZ and DG single cell suspension at a density of 2 x 10^4 cells/mL in SFM supplemented with 10 ng/mL EGF and 5 ng/mL bFGF. Seed SVZ and DG cells in uncoated 60 mm Petri dishes with a final volume of 5 mL/Petri dish.

5.3. Incubate SVZ and DG cells for 6–8 days and 10–12 days, respectively to form primary neurospheres, at 37 °C with 5% CO_2.

NOTE: Incubation days more than those mentioned can promote aggregation of neurospheres and higher levels of cell death in the center of the neurosphere.

5.4. When the majority of neurospheres have a diameter of 150–200 µm, perform the neurosphere passage.

NOTE: Passaging neurospheres when they do not have an appropriate diameter compromises all the next steps.

6. Passaging of neurospheres

NOTE: The following protocol can be applied to expand both SVZ and DG neurospheres.

6.1. To passage neurospheres, collect the SFM with growth factors containing neurospheres from the 60 mm Petri dish(es) and centrifuge for 5 min at 300 x *g*.

6.2. Discard the supernatant and resuspend the neurosphere pellet using a chemical dissociation kit (mouse) according to the manufacturer's instructions (**Table of Materials**).

NOTE: Observe the incubation times precisely as they are crucial for performance.

6.3. Centrifuge for 5 min at 300 x *g*, remove the supernatant and add 1 mL of SFM supplemented with 10 ng/mL EGF and 5 ng/mL bFGF.

6.4. Triturate up and down approximately 10x with a P1000 pipette to dissociate neurospheres.

6.5. Count the number of cells using a solution containing 0.2% Trypan blue and a hematocytometer.

6.6. Reseed cells at a density of 2 x 10^4 cells/mL in uncoated 60 mm Petri dishes.

6.7. Incubate SVZ and DG cells for 6−8 days and 10−12 days, respectively to obtain secondary neurospheres, at 37 °C with 5% CO_2.

NOTE: The self-renewal capacity of SVZ- and DG-derived NSPCs can be accessed by following protocol sections 5 and 6. For that, seed SVZ and DG cells at a density of 1.0 x 10^4 cells/mL (in uncoated 24-well plates) in growth SFM medium containing 5 ng/mL EGF and 2.5 ng/mL bFGF (low EGF/bFGF). Count the number of resulting primary and secondary neurospheres.

7. Storage of neurospheres

7.1. Collect the medium containing neurospheres (obtained from steps 5.3 and 6.7) from the 60 mm Petri dishes.

7.2. Centrifuge for 5 min at 300 x *g* and discard the supernatant.

7.3. Wash the cells 2x with 1 mL of HBSS (5 min at 300 x *g*).

7.4. Centrifuge for 5 min at 300 x *g*, discard the supernatant and store the pellet of neurospheres at -20 °C for molecular biology analysis.

8. PDL coating plate procedure

8.1. To prepare solution 1 (0.1 M borate buffer), weigh 3.92 g of boric acid and dilute in 400 mL of high purity water. Adjust the pH to 8.2 and make up to 500 mL with high purity water.

8.2. To prepare solution 2 (0.167 M borate buffer), weigh 10.3 g of boric acid and dilute in 900 mL of high purity water. Adjust the pH to 8.2 and make up to 1,000 mL with high purity water.

8.3. To reconstitute PDL (1 mg/mL in 0.1 M borate buffer), dilute 100 mg of PDL in 100 mL of solution 1.

8.4. Make aliquots of 10 mL to use immediately or freeze and store at -20 °C.

8.5. Under the laminar flow, add 1 coverslip per well and sterilize under UV light for 15 min.

8.6. Use the reconstituted PDL or thaw frozen reconstituted PDL.

8.7. Prepare the final solution of 100 µg/mL PDL in 0.167 M borate buffer by adding 10 mL of reconstituted PDL to 90 mL of solution 2.

8.8. Add the final solution to wells for a minimum of 2 h to overnight at 37 °C.

NOTE: For 24-well plates, add a volume of 500 µL in each well.

8.9. Remove the solution and wash 3x with high purity water.

8.10. Let the coverslips dry in the laminar flow hood.

8.11. Leave the multi-well culture plates at 4 °C.

9. PDL/Laminin coating plate procedure

9.1. On day 1, coat the plates with PDL as described in section 8.

9.2. On day 2, remove the PDL solution and wash 3x with high purity water. Let dry.

9.3. Prepare 5 µg/mL laminin in cold SFM devoid of growth factors.

9.4. Add dissolved laminin to the coverslips and incubate at 37 °C overnight.

NOTE: For 24-well plates, add a volume of 500 µL in each well.

9.5. Remove laminin using a pipette.

NOTE: Do not wash the coverslips from laminin.

9.6. Use immediately or store at -20 °C.

10. Poly-L-ornithine (PLO)/laminin coating procedure

10.1. Under the laminar flow, add one coverslip per well and sterilize under UV light for 15 min.

10.2. Add 0.01% PLO solution to each well for 20 min at room temperature (RT).

NOTE: For 24-well plates, add a volume of 500 µL in each well.

10.3. Remove the solution and wash 3x with sterilized 1x PBS. Let dry.

10.4. Prepare 5 µg/mL laminin in sterile 1x PBS.

10.5. Incubate for 2 h at 37 °C.

10.6. Remove laminin.

NOTE: Do not wash the coverslips from laminin.

10.7. Use immediately.

NOTE: Make sure that the coverslip is fully covered by the PLO solution by gently tapping the coverslip with a pipette tip. When shaken, the multi-well plates should make no sound.

11. Evaluation of neuritogenesis by generating a differentiated monolayer of cell

11.1. Collect media containing neurospheres from 60 mm Petri dishes (obtained from section 5) and centrifuge for 5 min at 300 x *g* at RT.

11.2. Discard the supernatant and dissociate the pellet of neurospheres in 1 mL of dissociation PBS (i.e., PBS without Mg^{2+}/Ca^{2+} and with EDTA [2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8.1 mM Na_2HPO_4 and 0.5 mM EDTA 4Na, at pH = 7.40]) by incubating for 15 min followed by mechanical dissociation. Alternatively, dissociate neurospheres using a chemical dissociation kit (mouse) (**Table of Materials**).

11.3. Centrifuge for 5 min at 300 x *g* at RT and discard the supernatant.

11.4. Resuspend the cell pellet in 1 mL of SFM devoid of growth factors.

11.5. Determine cell density using a hematocytometer.

11.6. Dilute the dissociated cell suspension in SFM devoid of growth factors at a density of 3,766 cells/cm^2 and plate cells on PDL coated glass coverslips in 24-well plates.

11.7. After 1−3 days, fix cells for immunocytochemistry against a protein of the cytoskeleton (see section 14).

12. Differentiation of neurosphere cultures

NOTE: Neurospheres obtained from cell expansion, either from primary or passaged neurospheres (obtained in sections 5 or 6) can be differentiated into cells from different neural lineages.

12.1. When neurospheres have a diameter of 150−200 µm, collect 25 µL of neurosphere suspension medium and plate on glass coverslips coated with 100 µg/mL PDL, in 24-well plates.

NOTE: To collect more neurospheres, gently rotate the Petri dish to concentrate the neurospheres in the center. Then, pipette from the center.

12.2. Put the plates in an incubator at 37 °C for 15 min so that neurospheres adhere to the substrate. Afterwards, add 500 µL of SFM devoid of growth factors (differentiative conditions).

12.3. After 24 h, replace the medium with fresh SFM devoid of growth factors.

12.4. Differentiate for different time points (e.g., 2 and 7 days in vitro, DIV2 and DIV7, respectively) with 5% CO_2 and 95% atmospheric air at 37 °C.

NOTE: Cell survival, proliferation and differentiation can be analysed using different cell assays.

13. Cell biology assays

13.1. Cell survival assay

13.1.1. Expose plated neurospheres to 3 µg/mL propidium iodide (PI) for 30 min before cell fixation in the incubator at 37 °C.

NOTE: PI is an autofluorescent agent that is only able to enter cells with compromised membrane integrity[8]. Other methods to analyse cell survival can be used such as caspase 3 staining or the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay.

13.2. Cell proliferation assay

13.2.1. Expose plated neurospheres to 10 µM 5-bromo-2'-deoxyuridine (BrdU) for 4 h before fixation in the incubator at 37 °C.

NOTE: BrdU is a synthetic thymidine analogue that can be incorporated during DNA synthesis in proliferating cells[9].

13.3. Cell differentiation assay

13.3.1. Expose 7-day-old plated neurospheres to 10 µM BrdU in the first 24 h, in the incubator at 37 °C.

13.3.2. Renew the SFM devoid of growth factors (differentiative conditions) and allow cells to develop in the absence of BrdU for the following 6 days until fixation.

NOTE: These pulse-chase experiments, by co-labelling with markers of mature neural cells, allow the evaluation of progenitor cells that differentiate into mature cells during the protocol.

14. Immunostaining of neurosphere cultures

14.1. Cell fixation

14.1.1. Prepare 4% paraformaldehyde (PFA) in 1x PBS and store at 4 °C or -20 °C.

14.1.2. Remove the SFM devoid of growth factors from wells and add, to each well, 500 µL of 4% PFA at 4 °C for 20 min at RT.

14.1.3. Wash 3x with 1x PBS, for 5 min each time, the coverslips containing differentiated neurospheres.

14.1.4. Store coverslips until use in 500 µL of 1x PBS at 4 °C.

NOTE: If the experiment does not have BrdU, skip to step 14.3.

14.2. Denaturation method (for BrdU experiments only)

14.2.1. Prepare 1 M HCl at 37 °C.

14.2.2. Rinse coverslips 3x in 1x PBS.

14.2.3. Permeabilize cells for 30 min in PBS containing 1% nonionic surfactant (e.g., Triton X-100).

14.2.4. Denature dsDNA with 1 M HCl pre-heated to 37 °C for 30–40 min at 37 °C (~300 µL/well).

14.2.5. Wash wells 4x with 1x PBS.

14.3. Permeabilization and blocking

14.3.1. Rinse coverslips in 1x PBS for 5 min.

14.3.2. Incubate for 1.5 h with 0.5% nonionic surfactant and 3% bovine serum albumin (BSA) in 1x PBS (~300 µL/well).

NOTE: For NeuN, use 6% BSA in 1x PBS.

14.4. Incubation and mounting

14.4.1. On day 1, without washing, incubate cells with primary antibodies (**Table of Materials**) in 0.1% nonionic surfactant and 0.3% BSA in 1x PBS in the incubation chamber (for 24-well plates use 20 µL/well). Leave coverslips incubating overnight at 4 °C light protected if antibodies are conjugated to a fluorophore.

14.4.2. On day 2, return coverslips to their respective wells and rinse 3x in 1x PBS for 5 min.

14.4.3. Counterstain with appropriate fluorescence conjugated secondary antibodies (dilution 1:200) and with 12 µg/mL Hoechst 33342 in 1x PBS for 2 h at RT and light protected in the incubation chamber (20 µL/coverslip).

14.4.4. Wash coverslips 3x in 1x PBS for 5 min.

14.4.5. Mount coverslips onto microscope slides using 5 µL/coverslip of fluorescence mounting medium.

14.4.6. Let coverslips air dry at RT, protected from light, for 1 day.

14.5. Microscopy

14.5.1. View and acquire images using a fluorescence microscope.

14.5.2. For each condition, use three replicates. Perform cell counts in five independent microscopic fields in each coverslip with a 40x objective (~100 cells per field).

15. Preparation of EGF and bFGF stock solutions

15.1. EGF stock solution

15.1.1. To reconstitute lyophilized EGF, dilute the product in high purity water to reach a final concentration of 20 µg/mL.

15.1.2. Aliquot and store in microtubes at -5 to -20 °C.

15.2. bFGF stock solution

NOTE: bFGF must be reconstituted with a solution of 10 mM Tris, pH 7.6.

15.2.1. Centrifuge the vial briefly before opening to bring the content to the bottom.

15.2.2. Prepare 50 mL of 10 mM Tri, pH 7.6. For that, weigh 60.57 mg of Tris ($(HOCH_2)_3CNH_2$) and dilute it in 40 mL of high purity water. Adjust the pH to 7.6 and make up to 50 mL with high purity water.

15.2.3. Prepare 10 mL of 0.1% BSA in 10 mM Tris, pH 7.6. For that, weigh 10 mg of BSA and dilute it in 10 mL of 10 mM Tris.

15.2.4. Filter solutions prepared in steps 15.2.2 and 15.2.3 with a 0.22 µm filter under a laminar flow hood.

15.2.5. Reconstitute 10 µg of bFGF in 1,000 µL of 0.1% BSA in 10 mM Tris with pH 7.6 to reach a final concentration of 10 µg/mL. Aliquot into microtubes at -20 °C for a maximum of 6 months.

REPRESENTATIVE RESULTS:

SVZ and DG neurospheres, obtained by using the NSA, are composed of undifferentiated cells, positive for Sox2, a transcription factor involved in the self-renewal capacity and positive for nestin, an intermediate filament protein expressed in NSPCs (**Figure 1A**). In addition, SVZ-derived neurospheres have larger dimensions than their DG counterparts (**Figure 1A**). Importantly, in differentiative conditions, SVZ- and DG-derived NSPCs migrate out of neurospheres forming a pseudomonolayer of cells (**Figure 1B**).

To access the self-renewal capacity, the cell pair assay is performed based on the expression of Sox2 and nestin which tends to disappear in dividing cells that start the differentiation process with a combination of a marker of the neuronal lineage namely, DCX. In both neurogenic regions, it is possible to observe the presence of $Sox2^{+/+}$/$nestin^{+/+}$/$DCX^{-/-}$ symmetrical divisions (self-renewal) (**Figure 2A1,B1**), $Sox2^{-/+}$/$nestin^{-/+}$/$DCX^{+/-}$ asymmetrical divisions (**Figure 2A1,B2**) and $Sox2^{-/-}$/$nestin^{-/-}$/$DCX^{+/+}$ symmetrical divisions (differentiation) (**Figure 2A2,B1**).

Passaging the neurospheres increases the yield of NSPCs; however, cell death at DIV2 changes with passaging. In fact, the percentage of PI-positive cells is increased with cell passage in SVZ (P0: 15.6% ± 1.2% vs P1: 19.2% ± 2.7% vs P2: 32.35% ± 0.14% vs P3: 39.6% ± 4.0%) and in DG (P0: 16.31% ± 0.95% vs P1: 32.1% ± 1.7% vs P2: 27.42% vs P3: 32.2% ± 3.1%) (**Figure 3**).

Neuritogenesis can be evaluated in neurons obtained from the differentiation of SVZ and DG NSPCs at the beginning of differentiation: DIV1 (**Figure 4A,D**), DIV2 (**Figure 4B,E**) and DIV3 (**Figure 4C,F**). In fact, as observed in **Figure 4**, the length and ramification of the neurites increases with differentiation.

Cell proliferation can be evaluated in SVZ- and DG-derived neurospheres. Comparing primary differentiated neurospheres at DIV1 from SVZ (**Figure 5A1**) and DG (**Figure 5A2**), the percentage of BrdU-positive cells is higher in SVZ than in DG (SVZ: 6.15% ± 0.64% vs DG: 3.27% ± 0.13%; $p < 0.05$; $n = 4$; **Figure 5A3**). Moreover, cell differentiation can also be accessed by combining BrdU staining with a mature maker such as neuronal nuclei (NeuN) that identifies mature neurons (**Figure 5B1,B2**). **Figure 5B3** shows that the percentage of proliferating progenitors that differentiate into mature neurons is similar in SVZ and DG (SVZ: 12.04% ± 1.581% vs DG: 13.56% ± 0.4831%; $p > 0.05$; $n = 4$).

The stemness and the multipotency of SVZ- and DG-derived NSPCs can be accessed using the NSA by evaluating the expression of different markers at different differentiation days (DIV2 and DIV7). Indeed, NSCs (nestin- and glial fibrillary acidic protein [GFAP]-double-positive cells) are present in both neurogenic regions (**Figure 6A,G**). These cells are able to differentiate into immature neurons (DCX-positive cells) (**Figure 6B,H**), mature neurons (NeuN-positive cells) (**Figure 6F,L**), oligodendrocyte precursor cells (neuron-glial antigen 2 [NG2] and platelet-derived growth factor receptor α (PDGFRα)- positive cells) (**Figure 6C,I**), mature oligodendrocytes (myelin

basic protein [MBP]-positive cells) (**Figure 6E,K**) and astrocytes (GFAP-positive cells) (**Figure 6D,J**).

Different substrates can be used to coat coverslips to form the pseudomonolayer of cells under differentiative conditions. As shown in **Supplementary Figure 1**, DG cells migrate more when the coverslips have extra-coating with laminin combined with PLO or PDL than with PDL alone (**Supplementary Figure 1B–H**). In fact, when PDL and laminin are used together as substrates (**Supplementary Figure 1C,G**), DG cells form a more confluent pseudomonolayer than SVZ cells for which PDL is used alone (**Supplementary Figure 1A,E**).

Importantly, these results demonstrate the potential of the NSA to evaluate the stemness and multipotency properties of NSCs derived from the two main neurogenic niches.

FIGURE LEGENDS:

Figure 1: Subventricular zone and dentate gyrus derived NSPC cultured as neurospheres or as pseudomonolayers. (**A**) Representative brightfield (**A1,A3**) and fluorescence (**A2,A4**) images of SVZ- and DG-derived neurospheres, where nuclei were stained with Hoechst 33342 (blue) and NSCs for Sox2 (green) and nestin (red). (**B**) Representative brightfield images of pseudomonolayers generated from SVZ- and DG-derived neurospheres under differentiative conditions.

Figure 2: The cell pair assay. Representative fluorescence images of cell pairs derived from a progenitor cell division. SVZ and DG nuclei were stained with Hoechst 33342 (blue), stem-like cells for Sox2 (red) and nestin (white) as well as immature neurons with DCX (green). Arrowheads in panels A1 and B1 indicate $Sox2^{+/+}$/$nestin^{+/+}$/$DCX^{-/-}$ symmetrical self-renewing divisions, arrows in panels A1 and B2 indicate $Sox2^{+/-}$/$nestin^{+/-}$/$DCX^{-/+}$ asymmetrical divisions, dashed line arrows in panels A2 and B1 show $Sox2^{-/-}$/$nestin^{-/-}$/$DCX^{+/+}$ symmetrical differentiating divisions.

Figure 3: Cell survival analysis with cell passaging. Quantitative analysis of PI-positive cells at DIV2 in DG- and SVZ-derived differentiated neurosphere culture, after 0, 1, 2 and 3 passages (P0–P3). Data is expressed as mean ± SEM, n = 1–8. PI = propidium Iodide.

Figure 4: Neuritogenesis analysis at DIV 1, 2 and 3. Representative confocal fluorescence images of neurites, identified by the βIII-tubulin signal, in SVZ and DG neurons at (**A,D**) DIV1, (**B,E**) DIV2, and (**C,F**) DIV3.

Figure 5: Cell proliferation assay. Representative confocal images of BrdU-positive cells at DIV1 in (**A1**) SVZ and (**A2**) DG. (**A3**) Quantitative analysis of BrdU-positive cells at DIV1 in DG- and SVZ-derived differentiated neurosphere culture. Data is expressed as mean ± SEM, n = 4. *$p < 0.05$ by t-test. Representative fluorescence images of BrdU- and NeuN-positive cells at DIV7 in (**B1**) SVZ and (**B2**) DG. Arrowheads indicate BrdU-/NeuN-positive cells. (**B3**) Quantitative analysis of BrdU-/NeuN-positive cells at DIV7 in both niches. Data is expressed as mean ± SEM, n = 4. BrdU: 5-bromo-2'-deoxyuridine, synthetic thymidine analogue.

Figure 6: Neural cell types present in SVZ- and DG-derived differentiated neurosphere culture. Representative fluorescence images of SVZ- and DG-derived cell types after 2 and 7 days of neurosphere differentiation (DIV2 and DIV7), where cell nuclei were stained with Hoechst 33342 (blue) and: (**A,G**) NSCs for GFAP (green) and nestin (red), (**B,H**) immature neurons for DCX (green), (**C,I**) oligodendrocyte precursor cells for PDGFRα (green) and NG2 (red), (**D,I**) astrocytes for GFAP (green), (**E,K**) mature oligodendrocytes for MBP (green), and (**F,L**) mature neurons for NeuN (red).

Supplementary Figure 1: Testing different substrates for neuropshere adherence and migration to form a pseudomonolayer. Representative fluorescence images of (**A,E**) SVZ-derived pseudomonolayer using poly-D-lysine as a substrate, (**B,F**) DG-derived pseudomonolayer using poly-D-lysine as a substrate, (**C,G**) DG-derived pseudomonolayer using poly-D-lysine with laminin as a substrate, and (**D,H**) DG-derived pseudomonolayer using poly-D-lysine with poly-L-ornithine as a substrate.

DISCUSSION:

In vitro systems of NSPCs allow a better understanding of the cellular and molecular mechanisms, which can be further validated in vivo. The NSA is a very powerful method to mimic physiological conditions due to their three-dimensional structure. Moreover, this culture system is also technically easier to culture[10], compared with other in vitro systems such as the monolayer culture system. Indeed, with the NSA, it is easy to control the exposed extrinsic cues during cell development, either during the expansion or the differentiation phase, by adding precise and variable amounts of factors of interest to the media as well as by culturing neurospheres with other cell types[6]. Furthermore, compared with monolayer cultures, in the NSA, it is possible to obtain a higher cell density from a small amount of tissue or with a small number of cells, allowing parallel studies to be performed, thus reducing the number of animals[1].

The NSA is the most common method to isolate and expand NSCs[11–13], can be used to estimate the number of precursor cells present in a given tissue sample[5] and the precursor cell frequency between different conditions. However, both neurospheres and monolayer cultures do not account for quiescence NSCs[14]. Moreover, the NSA has some limitations[11–13] and the resulting neurosphere frequency depends on many factors including the medium components, the dissection procedure, the dissociation process[11–13], and neurosphere aggregation[5]. Indeed, in a high-density culture, neurospheres tend to aggregate. Consequently, caution must be taken when estimating the number of precursor cells in a sample. To overcome the above limitations, isolated NSPCs can also be expanded and passaged in a monolayer[5,15]. Importantly, using NSA to compare precursor cell frequency between different conditions is very useful and accurate because all these limitations are implicit and similar among all conditions performed in the same experiment.

There are critical steps in the neurosphere culture that need attention. In the brain harvesting step, complete removal of the meninges and good isolation of the neurogenic niches are essential to maximize the purity and yield of NSPCs. During tissue dissociation, due to the proteolytic activity of trypsin, excessive use of trypsin or longer incubation times can lead to cell lysis. Furthermore, the day of the passage is critical to obtain a healthy population of neurospheres.

Passaging neurospheres with a diameter higher than 200 µm greatly affects the viability, proliferative and differentiative capacity of NSPCs. Importantly, longer cycles of passages more than 10 can increase genetic instability[6]. Furthermore, coating with PDL and PLD/laminin for SVZ and DG cells, respectively, is essential to ensure good cell migration out of the neurospheres without compromising the differentiation process. In terms of the immunocytochemistry analysis, longer incubation times with PFA can compromise staining by masking the antigens and increasing the background.

The NSA provides a powerful tool for providing a consistent and an unlimited source of NSPCs for in vitro studies of neural development and differentiation as well as for therapeutic purposes[16,17]. Indeed, this assay can be applied to genetic and behavioral models to further understand the molecular and cellular mechanisms involved in NSPC proliferation and differentiation[18,19]. This assay is also useful to test different drugs and compounds[20–22] as well as to perform genetic manipulations[19,23] to modulate NSC properties. In addition to immunocytochemistry, reverse transcription polymerase chain reaction and Western blot analysis can be performed to access RNA and protein expression, while electrophysiological studies and calcium imaging can be used to evaluate the function of the new-born neurons[21].

ACKNOWLEDGMENTS:

This work was supported by IF/01227/2015 and UID/BIM/50005/2019, projeto financiado pela Fundação para a Ciência e a Tecnologia (FCT)/ Ministério da Ciência, Tecnologia e Ensino Superior (MCTES) através de Fundos do Orçamento de Estado. R.S. (SFRH/BD/128280/2017, F.F.R. (IMM/CT/35-2018), D.M.L. (PD/BD/141784/2018), and R.S.R. (SFRH/BD/129710/2017) received a fellowship from FCT. The authors would like to thank the members of the bioimaging facility at Instituto de Medicina Molecular João Lobo Antunes for microscopy assistance.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Reynolds, B., Weiss, S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science*. **255** (5052), 1707–1710 (1992).
2. Azari, H., Rahman, M., Sharififar, S., Reynolds, B.A. Isolation and expansion of the adult mouse neural stem cells using the neurosphere assay. *Journal of Visualized Experiments*. (45) e2393 (2010).
3. Vescovi, A.L., Reynolds, B.A., Fraser, D.D., Weiss, S. bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells. *Neuron*. **11** (5), 951–66 (1993).
4. Rietze, R.L., Reynolds, B.A. Neural Stem Cell Isolation and Characterization. *Methods in Enzymology*. **419**, 3–23 (2006).
5. Walker, T.L., Kempermann, G. One Mouse, Two Cultures: Isolation and Culture of Adult Neural Stem Cells from the Two Neurogenic Zones of Individual Mice. *Journal of Visualized Experiments*. (84), e51225 (2014).
6. Jensen, J.B., Parmar, M. Strengths and Limitations of the Neurosphere Culture System.

Molecular Neurobiology. **34** (3), 153–162 (2006).
7. Xapelli, S. et al. Activation of Type 1 Cannabinoid Receptor (CB1R) Promotes Neurogenesis in Murine Subventricular Zone Cell Cultures. *PLoS ONE*. **8** (5), e63529 (2013).
8. Riccardi, C., Nicoletti, I. Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nature Protocols*. **1** (3), 1458–1461 (2006).
9. Nowakowski, R.S., Lewin, S.B., Miller, M.W. Bromodeoxyuridine immunohistochemical determination of the lengths of the cell cycle and the DNA-synthetic phase for an anatomically defined population. *Journal of Neurocytology*. **18** (3), 311–318 (1989).
10. Weinberg, D., Adams, C.F., Chari, D.M. Deploying clinical grade magnetic nanoparticles with magnetic fields to magnetolabel neural stem cells in adherent versus suspension cultures. *RSC Advances*. **5** (54), 43353–43360 (2015).
11. Azari, H., Sharififar, S., Rahman, M., Ansari, S., Reynolds, B.A. Establishing Embryonic Mouse Neural Stem Cell Culture Using the Neurosphere Assay. *Journal of Visualized Experiments*. (47), e2457 (2011).
12. Azari, H., Louis, S.A., Sharififar, S., Vedam-Mai, V., Reynolds, B.A. Neural-Colony Forming Cell Assay: An Assay To Discriminate Bona Fide Neural Stem Cells from Neural Progenitor Cells. *Journal of Visualized Experiments*. (49), e2639 (2011).
13. Reynolds, B.A., Rietze, R.L. Neural stem cells and neurospheres—re-evaluating the relationship. *Nature Methods*. **2** (5), 333–336 (2005).
14. Pastrana, E., Cheng, L.-C., Doetsch, F. Simultaneous prospective purification of adult subventricular zone neural stem cells and their progeny. *Proceedings of the National Academy of Sciences*. **106** (15), 6387–6392 (2009).
15. Conti, L., Cattaneo, E. Neural stem cell systems: physiological players or in vitro entities? *Nature Reviews Neuroscience*. **11** (3), 176–187 (2010).
16. Ghate, P.S., Sidhar, H., Carlson, G.A., Giri, R.K. Development of a novel cellular model of Alzheimer's disease utilizing neurosphere cultures derived from B6C3-Tg(APPswe,PSEN1dE9)85Dbo/J embryonic mouse brain. *SpringerPlus*. **3** (1), 161 (2014).
17. Pluchino, S. et al. Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. *Nature*. **422** (6933), 688–694 (2003).
18. Ogawa, Y., Kaizu, K., Yanagi, Y., Takada, S., Sakuraba, H., Oishi, K. Abnormal differentiation of Sandhoff disease model mouse-derived multipotent stem cells toward a neural lineage. *PLoS ONE*. **12** (6), e0178978 (2017).
19. Khacho, M. et al. Mitochondrial Dynamics Impacts Stem Cell Identity and Fate Decisions by Regulating a Nuclear Transcriptional Program. *Cell Stem Cell*. **19** (2), 232–247 (2016).
20. Soares, R. et al. Tauroursodeoxycholic Acid Enhances Mitochondrial Biogenesis, Neural Stem Cell Pool, and Early Neurogenesis in Adult Rats. *Molecular Neurobiology*. **55** (5), 3725–3738 (2017).
21. Rodrigues, R.S., Ribeiro, F.F., Ferreira, F., Vaz, S.H., Sebastião, A.M., Xapelli, S. Interaction between Cannabinoid Type 1 and Type 2 Receptors in the Modulation of Subventricular Zone and Dentate Gyrus Neurogenesis. *Frontiers in Pharmacology*. **8**, 516 (2017).
22. Xapelli, S. et al. Modulation of subventricular zone oligodendrogenesis: a role for hemopressin? *Frontiers in Cellular Neuroscience*. **8**, 59 (2014).
23. Kim, H.J. et al. Dynamin-related protein 1 controls the migration and neuronal differentiation of subventricular zone-derived neural progenitor cells. *Scientific Reports*. **5** (1), 15962 (2015).

Figure 1

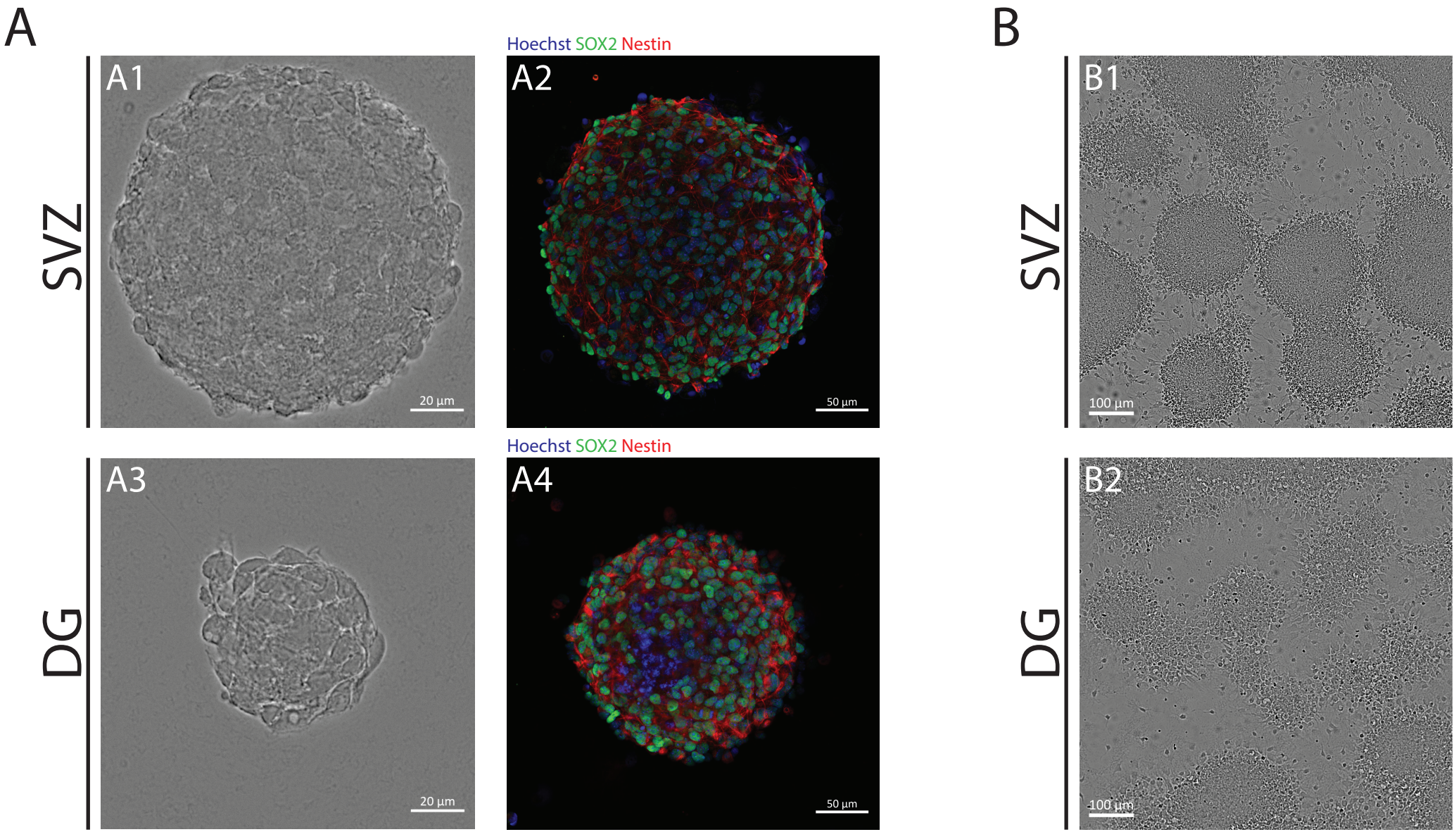


Figure 2

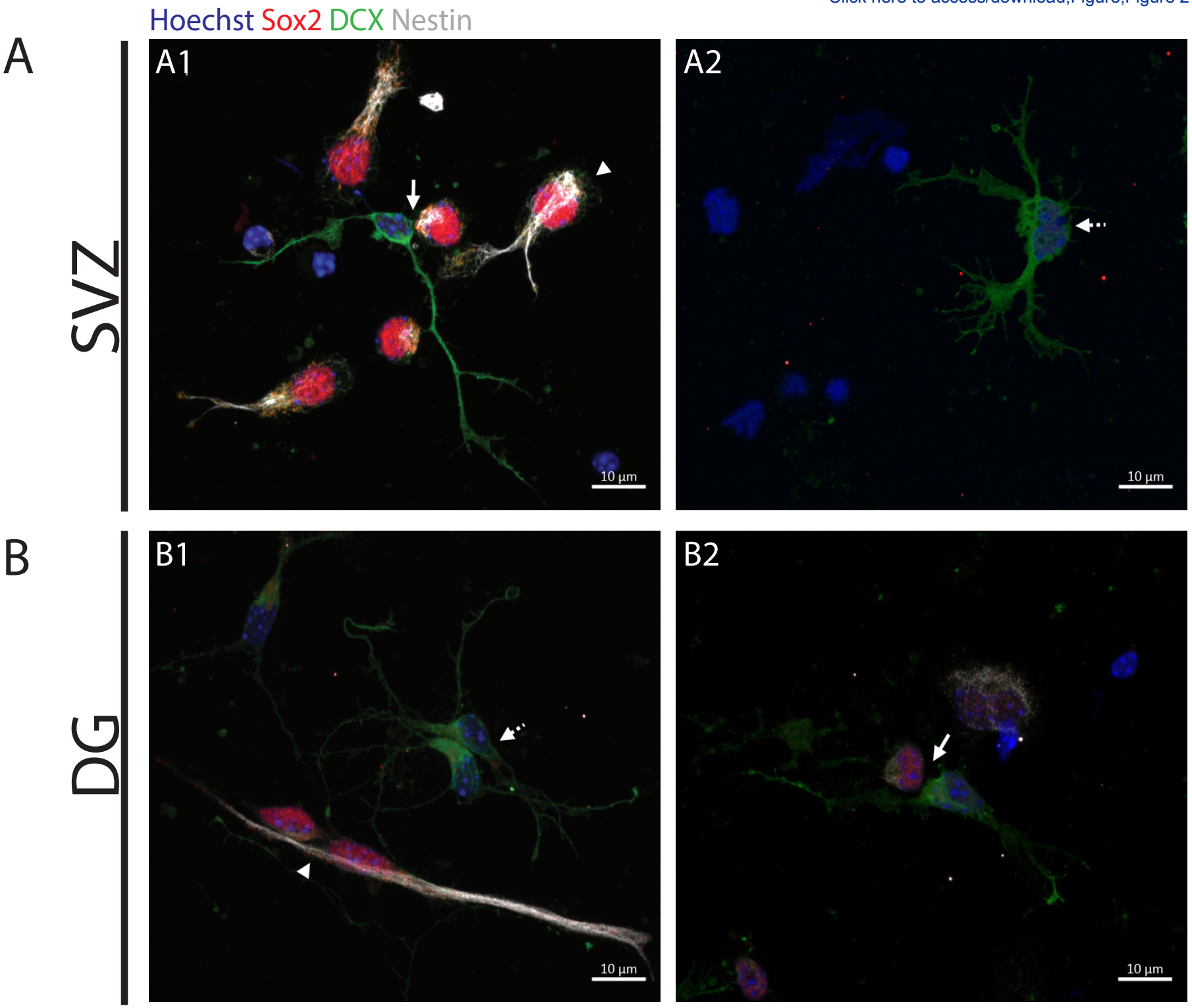


Figure 3

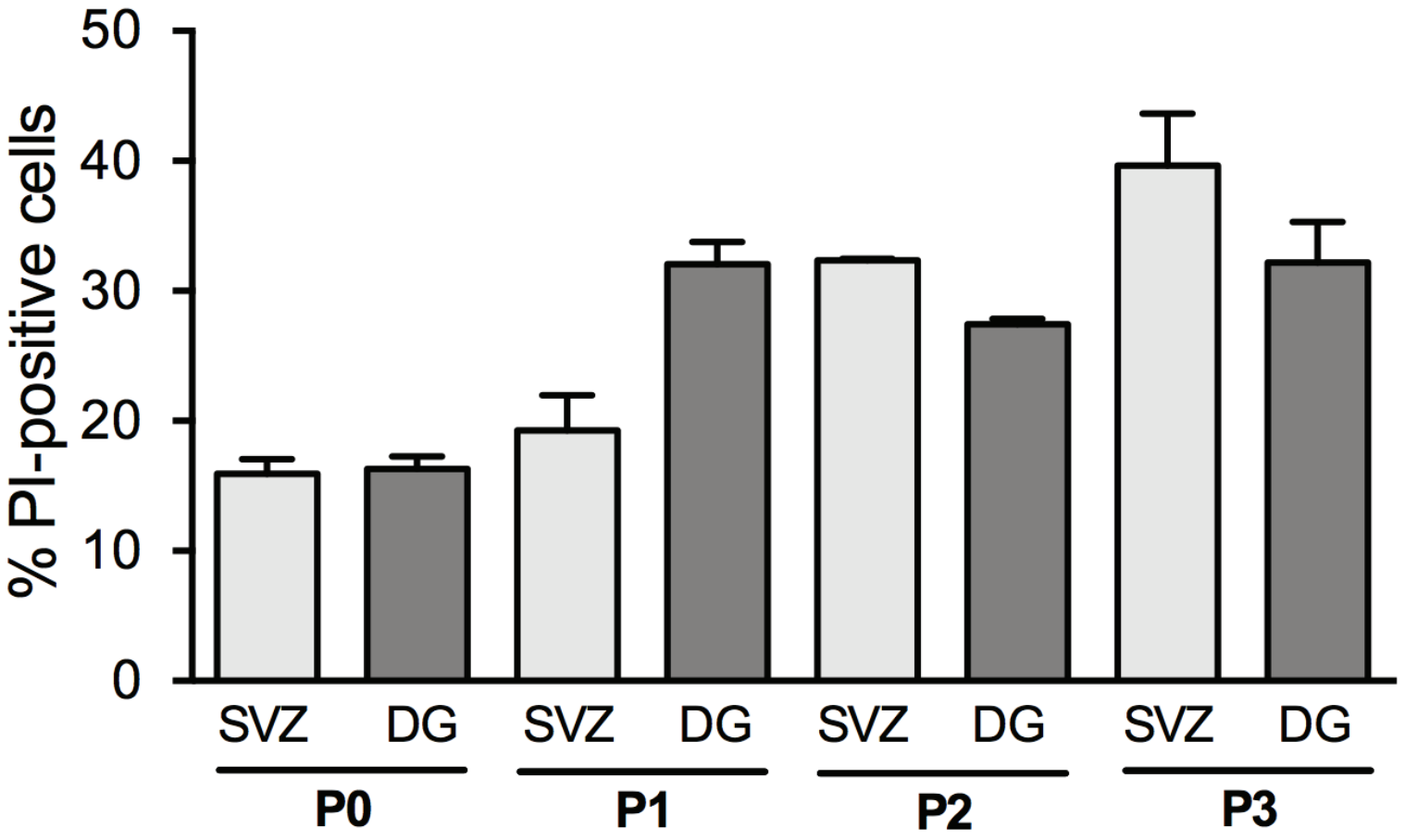


Figure 4

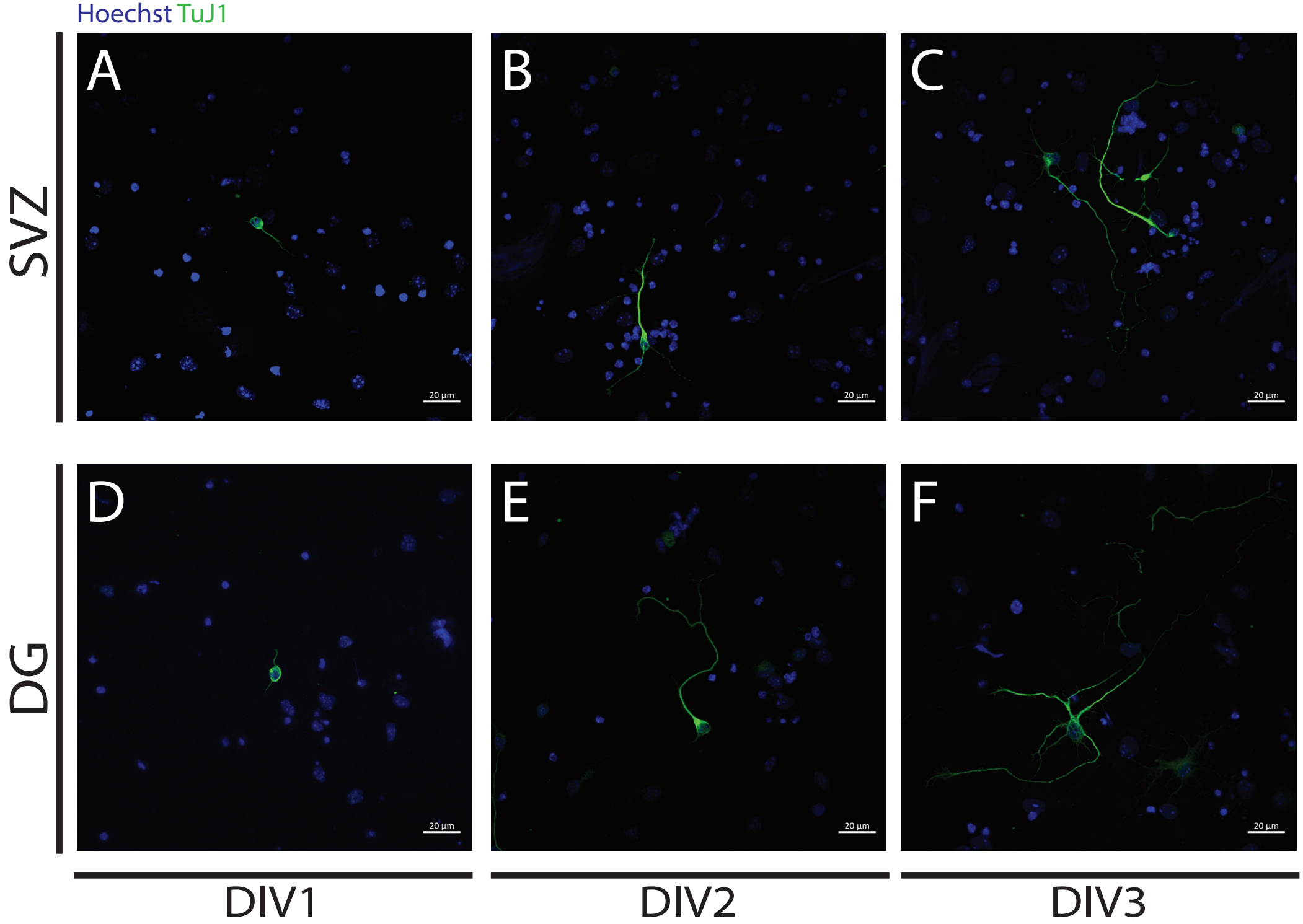
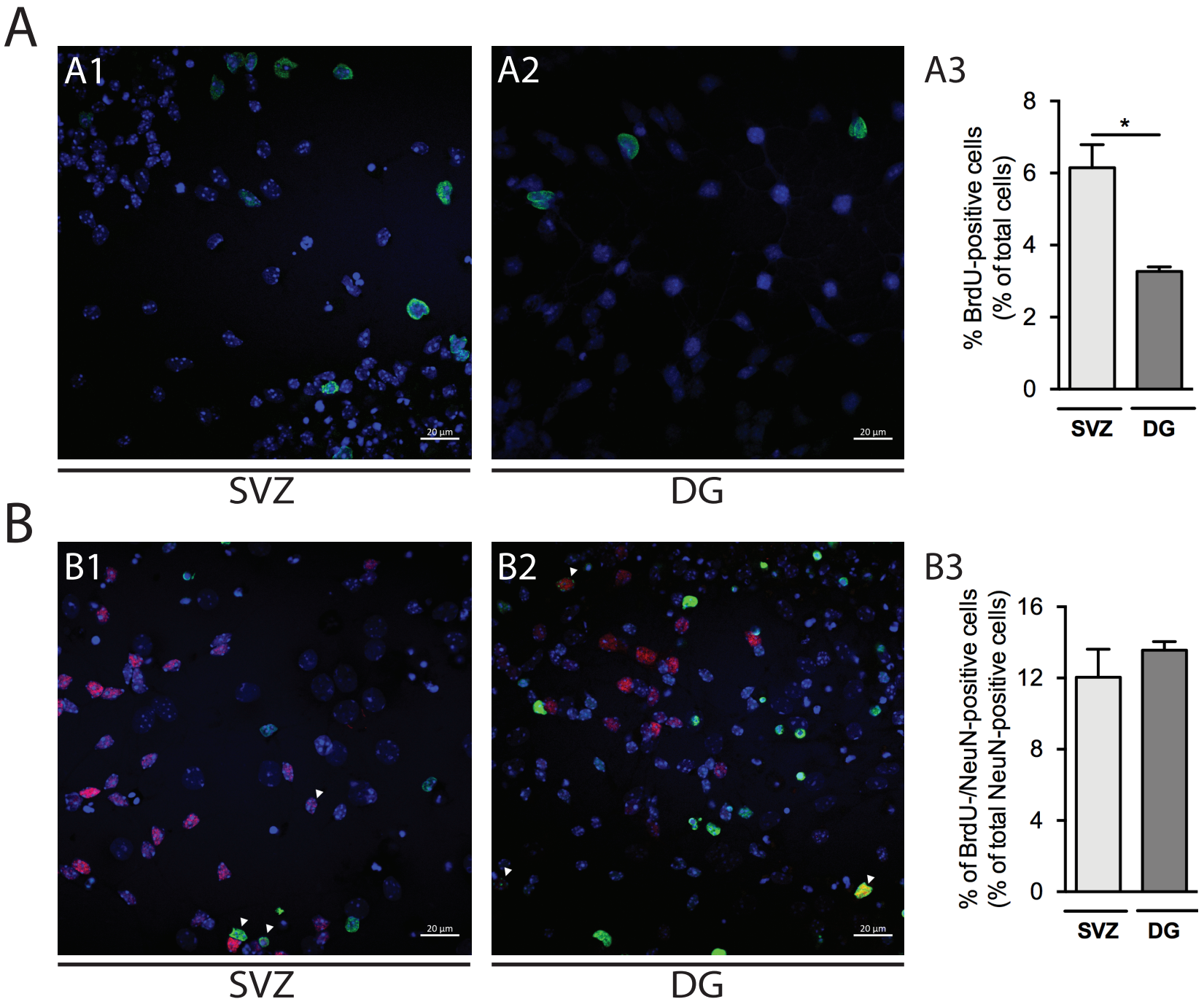
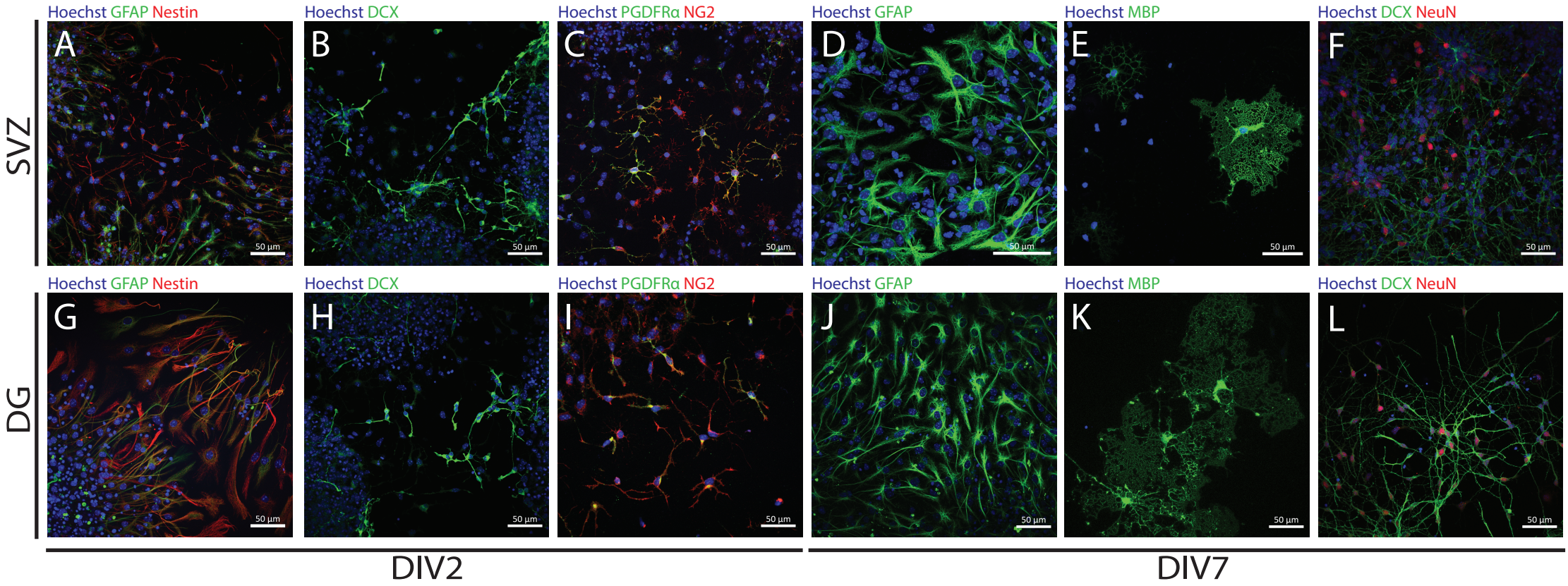


Figure 5





Name of Material/Equipment	Company	Catalog Number
0.05% Trypsin-EDTA (1X)	Gibco	25300-054
0.4% Trypan Blue solution	Sigma-Aldrich	T8154-20ML
12mm Glass coverslips	VWR	631-1577
15mL Centrifuge Tube	Corning	430791
5-bromo-2'-deoxyuridine	Sigma-Aldrich	B9285-1G
50 mL Centrifuge Tube	Corning	430829
70% Ethanol	Manuel Vieira & Cª (Irmão) Sucrs, Lda	UN1170
Adhesion slides, Menzel Gläser, SuperFrost Plus	VWR	631-9483
Alexa Fluor 488 donkey anti-chicken IgG (H+L)		
Alexa Fluor 488 donkey anti-rabbit IgG (H+L)	Life Technologies	A21206
Alexa Fluor 488 donkey anti-rat IgG (H+L)	Life Technologies	A21208
Alexa Fluor 568 donkey anti-mouse IgG (H+L)	Life Technologies	A10037
Alexa Fluor 568 donkey anti-rabbit IgG (H+L)	Life Technologies	A10042
Alexa Fluor 647 goat anti-mouse IgG (H+L)	Life Technologies	A21235
Anti-5-Bromo-2-Deoxyuridine	Dako	M0744
Anti-CD140a (PDGFRα) (rat)	BD Biosciences	558774
(rabbit)	Merck Milipore	AB5320
Anti-Doublecortin (rabbit)	Abcam	ab18723
Anti-Doublecortin (chicken)	Synaptic Systems	326006
Anti-Glial Fibrillary Acidic Protein (rabbit)	Sigma-Aldrich	G9269-.2ML
Anti-Myelin Basic Protein (rabbit)	Cell Signalling Technology	78896S
Anti-Nestin (mouse)	Merck Milipore	MAB353
Anti-Neuronal Nuclei (mouse)	Merck Milipore	MAB377
Anti-SOX2 (rabbit)	Abcam	ab97959
Anti-Tubulin β3 (rabbit)	BioLegend	802001
Axiovert 200 wide field microscope	ZEISS	
B-27 Supplement (50X), serum free	ThermoFisher	17504044
Boric Acid	Sigma-Aldrich	B6768-500g
Bovine Serum Albumin	NZYTech	MB04602
Cell counting chamber, Neubauer	Hirschmann	8100104
Cell culture CO_2 incubator	ESCO	CCL-170B-8

Corning Costar TC-Treated 24 Multiple Well Plate	Corning	CLS3524-100EA
di-Sodium hydrogen phosphate dihydrate	Merck Milipore	1.06580.1000
DMEM/F-12, GlutaMAX Supplement	ThermoFisher	31331028
Dumont #5 - Fine Forceps	FST	11254-20
Dumont #5S Forceps	FST	11252-00
Dumont #7 Forceps	FST	11272-30
Epidermal growth factor	ThermoFisher	53003018
Fibroblast growth factor	ThermoFisher	13256029
Filter papers	Whatman	1001-055
Fine Scissors - Sharp	FST	14060-09
Gillete Platinum 5 blades	Gillette	
HBSS, no calcium, no magnesium	ThermoFisher	14175053
Hoechst 33342	Invitrogen	1399
Hydrochloric acid	Merck Milipore	1.09057.1000 (1L)
Labculture Class II Biological Safety Cabinet	ESCO	2012-65727
Laminin	Sigma-Aldrich	L2020
McILWAIN Tissue Chopper	The Mickle Laboratory Engineering CO. LTD	MTC/2
Micro Spatula - 12 cm	FST	10091-12
Micro tube 0.5 mL	SARSTEDT	72.699
Micro tube 1.5 mL	SARSTEDT	72.690.001
Micro tube 2.0 mL	SARSTEDT	72.691
NeuroCult Chemical Dissociation Kit (Mouse)	Stem Cell	5707
Olympus microscope SZ51	Olympus	SZ51
Paraformaldehyde, powder	VWR	28794.295
Penicillin-Streptomycin	ThermoFisher	15140122
Petri dishes 60 mm	Corning	430166
Phosphate standard solutions, $PO_4$3- in water	BDH ARISTAR	452232C
Poly-D-Lysine 100mg	Sigma-Aldrich	P7886
Poly-L-ornithine solution	Sigma-Aldrich	P4957
Potassium chloride	Sigma-Aldrich	P5405-250g
Propidium iodide	Sigma-Aldrich	P4170-25MG
Sodium chloride	VWR	27800.360.5K

Sodium Hydroxide	Merck Milipore	535C549998
Triton X-100	BDH	14630
VWR INCU-Line IL10	VWR	390-0384

Comments/Description

Dilute at a ratio 1:500.
Dilute at a ratio 1:200.
Dilute at a ratio 1:200.
Dilute at a ratio 1:500.
Dilute at a ratio 1:1000.
Dilute at a ratio 1:200.
Dilute at a ratio 1:200.
Use 6% BSA in PBS 1X. Dilute at a ratio 1:400.
Dilute at a ratio 1:500.
Dilute at a ratio 1:200.

Set to 450 µm

Instituto de Medicina Molecular João Lobo Antunes

Dear Editor,

We would like to thank the editor and the referees for the thorough review of our submitted manuscript "Isolation and Expansion of Neurospheres from Postnatal (P1-3) Mouse Neurogenic Niches".

Regarding their suggestions we believe that we have improved the paper and we are convinced that, in its present form, the manuscript addresses the main criticisms raised by the reviewers.

We are now submitting the revised version of the manuscript with changes highlighted in grey.

We hope that you will find that this new version, together with the specific point-by-point replies made below to the editor and referees, are adequate for the publication of this study in JoVE.

Best regards,

Sara Alves Xapelli

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Response to Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

The manuscript was thoroughly reviewed, taking into account the mentioned issues.

2. Please revise lines 43-45, 74-77, 357-365, 418-420 to avoid textual overlap with previously published work.

We have rephrased the abovementioned sentences (changes highlighted in grey in lines 43-45, 76-79, 425-433 and 437-439).

3. All methods that involve the use of human or vertebrate subjects and/or tissue sampling must include an ethics statement. Please provide an ethics statement at the beginning of the protocol section indicating that the protocol follows the guidelines of your institution.

We have included an ethic statement at the beginning of the protocol (lines 90-94).

4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. Step 1 followed by 1.1, followed by 1.1.1, etc. Each step should include 1−2 actions and contain 2−3 sentences. Use subheadings and substeps for clarity if there are discrete stages in the protocol. Please refrain from using bullets, dashes, or indentations.

As suggested by the editor, the numbering of the protocol was changed to follow the JoVE Instructions for Authors. The changes are highlighted in grey. Due to the requested changes in the numbering, we have deleted sub-steps 2.1. and 2.2. identified as "SVZ Microdissection" and "DG Microdissection" respectively, moving these procedures to step 2 (lines 147-173). Moreover, we have also created a new step (step 13) which includes the different cell biology assays (line 340).

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5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: GlutaMAX, NeuroCult, Milli-Q, etc.

We have removed all commercial language from the manuscript. GlutaMAX was replaced by L-glumamine (line 100) and it was mentioned in the Table of Materials. We now only refer NeuroCult in the Table of Materials. We have Milli-Q water replaced by high purity water (lines 258, 259, 261, 262, 273, 282, 418, 426 and 427).

6. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

7. Lines 91, 97: Please list an approximate volume of medium to prepare.

Regarding the Editor's concern about the appropriate volume of growth medium and dissection medium to be prepared at the day of the culture, it is difficult to define this in the protocol since it depends on the number of pups, and consequently the amount of SVZ and DG cells to be obtained from the dissection. However, we have now mentioned a range of volumes to be prepared (lines 104-106 and 110).

8. Line 105: How large is the petri dish?

The diameter of the petri dish is 60 mm and the height is 15 mm. Moreover, the actual inside growth surface diameter is 51.4 mm and the approximate growth surface area is 21 cm^2. This information was added in line 117.

9. Line 107: Please specify the euthanasia method.

The Editor raised an important question about the euthanasia method. In this protocol, we use the decapitation method as described in step 2.2..

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10. Line 130: Please specify the pore size of the tissue paper.

Regarding the pore size of the tissue paper, it is 11 μm. This information was added in line 143.

11. Lines 134, 142, etc.: Please specify the surgical tools used here.

We have now specified in the manuscript the surgical tools needed for this protocol (lines 147, 151, and161).

12. Line 173: How to obtain the pellet? Is centrifugation used here?

When we mention the word "pellet" we are referring to the tissue obtained from the SVZ or DG dissection, obtained in the previous steps (step 3.1. and 3.2.). Therefore, to be more understandable, we changed the word "pellet" to "digested tissue" (line 185).

13. Line 227: Please specify incubation time.

Regarding the incubation time needed to obtain secondary neurospheres, it is similar to that required to form primary neurospheres, mentioned in line 216. As suggested, we now specify that information in the lines 240 and 241.

14. Line 236: Neurospheres from which step? Please specify.

Concerning the storage of neurospheres, this procedure can be applied in primary or passage neurospheres. Therefore, the neurospheres that are collected can be obtained after performing steps 5.3. or 6.7.. This information is now specified (line 249).

15. Line 237: What happens after centrifugation, discard the supernatant? Please specify.

In fact, it is not clear which procedure should be performed after the centrifugation. Indeed, the supernatant which corresponds to the growth medium has to be removed after the centrifugation to keep just the pellet (neurospheres). To be clearer, this information is now specified in line 251.

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16. Lines 276, 282, 288, 323: At what temperature? Please specify.

PI (line 276 of the original manuscript) and BrdU are incubated (line 282 and 288 of the original manuscript) at 37°C. These procedures are performed in cell cultures and that are maintained in culture after the exposure with PI or BrdU. Therefore, all the incubations are performed at 37 °C with 5% CO2 and 95% atmospheric air. The temperature was added in the sentences of the mentioned lines.

Concerning the primary antibodies, they are incubated at 4°C, as is now mentioned in line 396 of the revised manuscript.

17. Line 289: Please specify the medium used here.

Since, we will evaluate the differentiation capacity of the cells, the medium is the SFM devoid of growth factors. This is now mentioned in line 359.

18. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

As suggested by the editor, all the protocol steps were reviewed to have 2-3 action and maximum of 4 sentences per step.

Steps 7.4. and 7.5. were combined into one step (lines 253 and 254).

Steps 10.1.2, 10.1.3. and 10.1.4 in the original manuscript were combined into one step which now is the step 14.1.2. (line 370).

The first two steps of the protocol of the immunostaining of neurosphere culture of day 2 (10.4.1. and 10.4.2.) were also combined into the step 14.4.3 (line 400).

Step 10.4.6. of the original manuscript was deleted because this information is repeated in step10.4.7.

19. Please include single line spacing between each numbered step or note in the protocol.

We have adjusted the spacing.

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20. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

Concerning the steps to be featured in the video, we guarantee that we have them highlighted in yellow.

21. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

Regarding the highlighted steps to be included in the video, we ensure that the guidelines stated by the editor were followed, including the cohesive narrative with a logical flow between the steps and the presence of at least one action written in the imperative voice per step.

22. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Concerning the details of the steps to be featured in the video, we ensure that all the details are included in the highlighted text.

23. Table of Materials: Please remove any ™/®/© symbols.

We have removed these symbols from Table of Materials.

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Response to Reviewer 1:

The protocol described by the authors doesn't provided new information about how to culture and work with neurosphere cultures. It has been published by several groups how to make neurospheres cultures and there are several protocol manuscripts, even on Nature protocols describing this procedure in much detail. Also, one of the main weaknesses of this submission is the lack of a video showing the procedure, something that might be useful for most researchers interested in this particular type of cell culture.

Although there are several publications regarding the protocol of neurospheres, in our humble opinion our protocol brings new detailed information since we describe not only the neurosphere culture but also its applications including assays to evaluate the NSC properties: self-renewal, proliferation and multipotency as well as cell viability in different contexts. Unlike other published papers with this methodology, our protocol also integrates the test of different substrates for neurosphere adhesion and migration of the cells to form a pseudomonolayer (Supplementary figure 1).

Regarding the video, we have highlighted in yellow the steps (from the harvesting of postnatal mouse brain and SVZ/DG Microdissections to the Passaging of the neurospheres) which will be filmed after the final approval of the written manuscript.

Response to Reviewer 2:

Manuscript Summary:
The authors describe a protocol for isolating and propagating SVZ and DG progenitor cells.

Major Concerns:
None. I assume that the micro-dissections will be filmed by the Jove team since illustrations about the microdissections are missing in the Figures.

This part of the protocol was selected by us to be filmed by the JoVE team since this procedure involves many details that should be followed to guarantee an efficient isolation of the SVZ- and DG-derived NSPCs. In the text we have highlighted in yellow the steps (from the harvesting of postnatal mouse brain and SVZ/DG Microdissections to the Passaging of the neurospheres) which will be filmed after the final approval of the written manuscript.

Minor Concerns:
Some minor orthographic errors such as line 519 should read overcome instead of overcame.

The word "overcame" was changed to "overcome" (line 557).

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Response to Reviewer 3:

Manuscript Summary:
This could be a useful protocol in the list of existing protocols for the neurosphere assay, but careful editing and addition of missing information is necessary:

Major Concerns:
1) On page 3, line 165, for tissue dissociation, authors mention that they use a final concentration of 5%-10% of Trypsin-EDTA 0.05% to incubate for approximately 15 min at 37 °C. However, the concentration of trypsin EDTA is very important to decide how long to incubate. Please explain the use of different concentrations of Trypsin-EDTA for dissociation.

We have including the range of 5-10% of Trypsin because the final concentration of Trypsin-EDTA to dissociate the SVZ and DG tissue depends on the number of pups and therefore the amount of tissue that is obtained.

2) On page 4, line 202-203, authors describe " Incubate SVZ and DG cells for 6-8 days and 10-12 days, respectively to form primary 203 neurospheres, at 37°C with 5% CO2. " Were additional EGF and bFGF added to the dish and were the dishes rotating while incubating? Without enough growth factors and keeping the dish still in the incubator during the culture it is difficult to keep the neurospheres floating.

Regarding to the reviewer's question about the presence of additional EGF and bFGF in the 60mm-petri dishes, no additional growth factors are added in the dishes during neurosphere formation. When the cells are diluted in the SFM in the step 5.2., the medium already contains the proper concentration of growth factors needed to promote the proliferation of SVZ- and DG-derived NSPC for 6-8 days and 10-12 days, respectively. Moreover, during incubation, SVZ and DG petri dishes are not rotating in the incubator because this could induce an aggregation of the neurospheres precluding the formation of single neurospheres. Furthermore, neurospheres have already a spontaneous locomotion[1].

With these conditions, it is possible to obtain floating neurospheres as observed in Figure 1 of the Results section. Beyond the proliferative capacity of these cells, it is also not used any coating subtract in the petri dishes, preventing any cell adhesion and subsequently promoting the floating of the cells. This protocol (without adding extra growth factors or rotation) is well-established and used by different labs.

3) On page #5, line #235, the authors describe a method to freeze and store neurospheres in -20C. However, it is not clear if the neurosphere pellets were stored as such or in freezing media. Also, it is not clear how to recover those frozen neurospheres for further proliferation and differentiation. A protocol for recovering frozen neurospheres is missing.

The Reviewer raised an important question regarding the storage of the neurospheres. As mentioned in the last step of the section 7, what is stored is just the pellet of cells and we don't add any freezing media, and therefore there is

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no cell viability preventing the recovery of neurospheres. The stored cells are intended for DNA, RNA and protein extraction. With these procedures, different markers of proliferation and differentiation could be evaluated. For that, the cells have to be slowly thawed on ice.

4) On page 5, line 246-249, authors described two dissociation methods. One to dissociate the pellet of neurospheres in 1 mL of dissociation PBS. Authors gave the detailed components of dissociated PBS, but didn't describe the dissociation procedure. I assume it is mechanical dissociation which they should describe.

Concerning the dissociation procedure included in the protocol to evaluate the neuritogenesis, the dissociation is mechanical. To be clear, we included this information in the revised manuscript (line 312).

5) Authors mention on page 9, line 421, that SVZ neurospheres are larger compared to neurospheres from DG. However, readers may not appreciate such a difference as presented in figure 1 because of different scale bar sizes. I suggest the authors keep a) scale bars at the same size in both images; and b) zoom settings the same for SVZ and DG so that their point will be clear.

Regarding the scale bars of the Figure 1, we agree with the Reviewer about the difficulty to compare the size of SVZ and DG neurospheres having different scale bars. Therefore, we have changed the scale bar in the new images A1 and A3 of Figure 1. Regarding images A2 and A4 also of the Figure 1, we have replaced them by new images because they were acquired with different number of tiles, being impossible to have the same scale bar.

6) on page 9 line 422-424, authors make the conclusion that "Importantly, in differentiative conditions, SVZ- and DG-derived NSPCs migrate out of the neurosphere forming a pseudomonolayer of cells composed of different cell types (Figure 1B). From the figure 1B, I can't draw that conclusion. The rationale for coming up with their conclusion should be provided.

The Reviewers concern is valid because in the figure 1B it is just visible the migration of the cells out of the neurosphere and not specifically the presence of different cell types in the periphery. Therefore, we have re-phrased this sentence in the revised manuscript by deleting the last part (lines 440-442).

The presence of different cell types in the pseudomonolayer can be observed in figure 6 of the revised manuscript, where we show a pseudomonolayer of cells formed by different cell types including NSCs, oligodendrocyte precursor cell, myelinating oligodendrocytes, immature and mature neurons as well as astrocytes.

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Minor Concerns:
Page #1, line #61: Epidermal growth factor and not 'ependymal'
Page #1, line #62: Fibroblast growth factor and not 'Fibroblastic'
Page #2, line #131: Suggest authors provide Tissue Chopper information

We have corrected the name of the growth factors (line 63).

We have added the information regarding the Tissue Chopper (line 143 and table of materials).

Response to Reviewer 4:

Manuscript Summary:
Here the authors presented well described protocols for isolation and culturing of neural stem cells. The title is informative and contains important search words. Manuscript is well written and all procedures are clearly explained.

Major Concerns:
no

Minor Concerns:
Since this is methods manuscript in the Abstract the important information about key methods should be added, like basic design, the age of mice, duration of incubation etc.

Regarding the Abstract, the information asked by the Reviewer was added, namely the age of the mice (P1 to 3), the incubation period (which is 2-7 days, depending on the degree of differentiation that we want to study), as well as potential applications of the NSA (lines 49-56).

Please explain, why do you use bFGF without heparin, which protect bFGF from degradation at +37 (G. Chen et al., 2012. Stem Cell 30 (4):623-630)

Concerning the use of bFGF without heparin, this procedure was based in our previous studies[2, 3] and others[1, 4]. Although heparin is known to help the stabilization of bFGF we have successfully obtained neurospheres by using bFGF without heparine.

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In step 7, (Storage of Neurospheres) a question: why the neurospheres were frozen without DMSO, which routinely added as cryoprotectant, and why the authors recommend store the cells at -20 C? Did the authors check viability of the cells after re-freezing?

We store the neurosphere pellets, without adding any cryoprotectant because the stored cells are intended for molecular biology analysis (line 254). We have also addressed this question to Reviewer #3.

In the results concerning step 8: It will be good, but optional, add some pictures that will illustrate forming neurites.

As suggested, we have added pictures of the neurites at DIV 1, 2 and 3, in both neurogenic regions. To identify them we performed an immunocytochemistry against the protein βIII-Tubulin . The images are in the new Figure 4.

In step 9.3. to analyze differentiation fate, the authors offer use BrdU at DIV1 and then check expression of markers of mature cells at DIV7 in BrdU positive cells, but do not provide any illustration.

The Reviewer raised an important question about the results of the combination of BrdU with other mature markers regarding to the Cell Differentiation Assay. Therefore, we have now added in Figure 5 of the revised manuscript a graph (and representative images) showing the percentage of BrdU-positive/NeuN-positive cells divided by the total amount of NeuN-positive cells at DIV7. Importantly, with this result we can estimate the number of proliferating progenitors that differentiate into mature neurons. Moreover, in Figure 5, we have also added representative images of the graph A3 already present in the original manuscript (% of BrdU-positive cells at DIV1).

Response to Reviewer 5:

Manuscript Summary:
In this study the authors describe a protocol for the generation, expansion and differentiation of neural stem cells from the early postnatal subventricular zone (SVZ) and subgranular zone (SGZ), the two neurogenic niches described in the murine brain.

Major Concerns:
1. Neurosphere-forming assays have been widely used to identify stem cells based on their capacity to evaluate self-renewal and differentiation at the single cell level in vitro, mainly in the adult neurogenic niches. Thus authors should better clarify in the manuscript that all these experiments are performed in postnatal day 1-3 and not in adult NSCs. Also in the title.

We have reviewed all the sections of the document and we have now included in the revised manuscript the missing information in the title, introduction (line 84) and protocol (lines 115 and 210).

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2. Is still present in the field that the relationship between in vivo progenitors and neurosphere-initiating cells needs some refinement (Doetsch et al., 2002; Reynold sand Rietze, 2005; Pastrana et al., 2011). For example, it is shown that the neurosphere assay does not provide an accurate read-out of the number of stem cells in vivo, mainly because both transit amplifying progenitors and GFAP+ cells form neurospheres and can be serially passaged as multipotent stem cells. Moreover, the neurosphere assay likely does not detect quiescent stem cells as the purified population containing putative quiescent stem cells from the SVZ does not give rise to neurospheres (Pastrana et al., 2009). It is crucial to describe limitations of this assay and the critical points that researchers have to keep in mind before extrapolating results from the in vivo context. The authors should mention all these points in the introduction and discussion.

We are in agreement with the referee concerning the limitations of the neurosphere model. Therefore, while writing the manuscript we always mentioned NSPCs and not just NSCs since both GFAP+ and transit amplifying progenitor cells are able to generate neurospheres. In fact, unlike what happens *in vivo*, *in vitro* we cannot distinguish between type B and type C cells.

Regarding the fact that this method does not detect the presence of quiescence stem cells, since they do not proliferate under the presence of growth factors, we have added a new sentence in the revised manuscript (lines 551 and 552).

3. Sox2 is also expressed in differentiated cells thus in the cell-pair assay to study cell fate the immunocytochemistry against Sox2 in cell pairs should be combined with other markers such as Nestin or differentiated markers (DCX and NG2).

Concerning the question that Sox2-positive cells can be differentiated cells, we are in agreement with this observation. Therefore, we performed again the cell pair assay not only with Sox2 but also with Nestin and the differentiated marker DCX, as was suggested by the Reviewer. With this approach, we have observed symmetric pairs formed by two Nestin- and Sox2-positive cells which are negative for DCX (Figure 2A1 and B1), symmetric pairs composed by two DCX-positive cells, negative for Nestin and Sox2 (Figure 2A2 and B1) as well as asymmetric pairs with one Nestin- and Sox2-positive cell that is negative for DCX and the other cell positive for DCX and negative for Nestin and Sox2 (Figure 2A1 and B2), in both neurogenic niches. Therefore, we have changed the information related with the Figure 2 in the Results section (lines 445-449) as well as in the legend of the Figure (lines 500-503). Importantly, we have also added the Nestin and DCX markers to the protocol of the cell-pair assay in section 4 (lines 205-206).

4. For evaluation of neuritogenesis why the authors only incubate the cells for 1-3 days? Longer periods of cultures will favor neurite formation.

Regarding the neurite formation, we have differentiated cells from 1-3 DIV since with longer periods the network is so big making it almost impossible to

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distinguish different neurons. Moreover, with higher differentiation times the neurites are very long being difficult to follow them.

5. Cell survival assay can be improved combining the PI staining with a immunocytochemistry for activated Caspase3 or the staining with DAPI to also count apoptotic bodies.

Concerning the evaluation of cell death, we totally agree with the Reviewer regarding the combination of the PI staining with another method. Beyond activated Caspase3 and the staining with DAPI to access cell apoptosis, we can also do the terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay[5]. We have added in the manuscript an extra note with these alternative approaches to access cell survival (lines 346-348).

Minor Concerns:
In 11.3, 11.4 and 11.5 authors show different coating procedures (PDL, PDL/Laminin and PLO/Laminin) however these three coatings are not explained or defined in the experiments.

Concerning the section related with the three different coatings, these were explained in the Supplementary information. However, we have now moved them to the main manuscript. The PDL coating plate proceduree is in sections 8, the PDL/laminin coating plate procedure is in section 9 and the Poly-L-Ornithine (PLO)/Laminin coating procedure is in section 10.

In Figure 1A, Nestin immunostaining is not clearly seen. In Figure 1B, please indicate the number of days of differentiation.

Regarding to the images of the Figures 1A and 1B, they were replaced to address the question 5 of the Reviewer 3. In the final images, we ensure that the Nestin immunostaining is clearly seen. These images correspond to neurospheres in proliferative conditions with 0 days of differentiation.

In Figure 5, markers in red (Nestin, NG2 and NeuN) are not clearly seen.

We have updated Figure 5 (now Figure 6) so that the staining for Nestin, NG2 and NeuN markers are clearly seen (new images A, F, G and I).

References

1. Singec, I. *et al.* Defining the actual sensitivity and specificity of the neurosphere assay in stem cell biology. *Nature Methods*. **3** (10), 801–806,

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doi: 10.1038/nmeth926 (2006).

2. Soares, R. *et al.* Tauroursodeoxycholic Acid Enhances Mitochondrial Biogenesis, Neural Stem Cell Pool, and Early Neurogenesis in Adult Rats. *Molecular Neurobiology*. **55** (5), 3725–3738, doi: 10.1007/s12035-017-0592-5 (2017).

3. Xapelli, S. *et al.* Modulation of subventricular zone oligodendrogenesis: a role for hemopressin? *Frontiers in cellular neuroscience*. **8**, 59, doi: 10.3389/fncel.2014.00059 (2014).

4. Torrado, E.F., Gomes, C., Santos, G., Fernandes, A., Brites, D., Falcão, A.S. Directing mouse embryonic neurosphere differentiation toward an enriched neuronal population. *International Journal of Developmental Neuroscience*. **37**, 94–99, doi: 10.1016/j.ijdevneu.2014.07.001 (2014).

5. Xapelli, S. *et al.* Activation of Type 1 Cannabinoid Receptor (CB1R) Promotes Neurogenesis in Murine Subventricular Zone Cell Cultures. *PLoS ONE*. **8** (5), e63529, doi: 10.1371/journal.pone.0063529 (2013).

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Supplementary Figure 1

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