Title: Unbiased stereological method to assess proliferation throughout the subependymal zone

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Running Head: Unbiased stereological SEZ proliferation analysis.

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Summary

The subependymal zone (SEZ), frequently named as adult subventricular zone (SVZ), is a niche of adult neural stem and progenitor cells that lines a large extension of the lateral ventricles of the brain. The majority of the studies do not analyze the SEZ throughout its entire extension. Instead, studies of cell populations within the SEZ typically focus their analysis on a narrow space between specific bregma coordinates that provides a perspective of only a small portion of the SEZ. We have previously proposed a standard division for the SEZ at the anterior-posterior and dorsal-ventral axes based on external brain anatomical hallmarks (1). Herein, we describe in detail the procedure and a stereological approach that can be used to obtain an unbiased estimation of the SEZ cell proliferation under physiological and pathological conditions. This approach takes into consideration clear SEZ anatomical divisions, both on the anterior-posterior and dorsal-ventral axes, which will standardize future studies on the SEZ.

1. Introduction

In recent years the number of manuscripts focusing on the subependymal zone (SEZ), also named adult subventricular zone (SVZ), increased sharply. A substantial portion of these studies relies on estimations of the SEZ cell proliferation. Recently, a growing body of evidence indicates that the adult neural stem cells (NSC) and its progeny are highly variable along the SEZ niche (2, 3). Thus, the estimation of the proliferation rates along the SEZ should take into account these topographic gradients. The estimation of cell proliferation is a valuable tool to analyze the SEZ stem cell niche dynamics under determined conditions; for instance, in response to stroke and in neurodegenerative diseases as well as in response to specific stimuli, such as the intraperitoneal or intracerebroventricular administration of molecules/factors, and in transgenic mice (4-7). There are many different approaches to estimate cell proliferation in the SEZ, including estimation of total number of proliferating cells using optical fractionator method (8, 9), proliferating cells per area (10), per volume (11) or per section (12).

Stereology is, by definition, the three dimensional interpretation of planar sections of materials or tissues, and it aims to quantify properties of 3D objects from serial 2D sections of the sample (13). When analyzing SEZ cell proliferation, in order to get an unbiased stereological methodology, one must follow two key principles: 1) systematic uniform random sampling, i.e., the first section to be analyzed is selected randomly, and the subsequent sections must be apart from each other at consistent intervals, 2) that the sample has no constant pattern, i.e., there is no repetitive layer patterning in the object under analysis, and 3) the researcher performing this analysis must be blind to the experimental groups, i.e., the person who is analyzing the sections should not know the identity of the experimental groups being examined. After randomly selecting the sections, that may either comprise the entire SEZ or just its anterior, intermediate, posterior and post-posterior division (as described in Falcão et al, 2012) (1), the SEZ area is estimated for each section and the total number of proliferating cells [5-bromo-2'-deoxyuridine (BrdU) or Ki67 positive cells] are counted within the drawn area. The optical fractionator method estimates the total number of cells (or alternatively proliferating cells) from the number of cells estimated in a systematic randomly sampled set of unbiased virtual counting spaces covering the entire region of interest, i.e. the SEZ area previously drawn, with uniform distance between counting spaces in directions X, Y and Z. The later method is not the most indicated, and instead every proliferating cell should be counted, since the proliferative rates within a single SEZ section are extremely heterogeneous (1) and thus the optical fractionator method could provide estimation with low precision.

Of notice, the SEZ areas indicated above vary according to the position in the anterior-posterior axis and, consequently, the number of cells (total and proliferating) also changes according to their relative position in the SEZ.

2. Materials

2.1 BrdU preparation for proliferation assessment

The BrdU dose commonly used to assess proliferation in the SEZ, both for mice and rat is 50 mg/Kg. In order to inject twice the volume equivalent to the body weight of the rat prepare a solution of 25mg/mL of BrdU (Sigma, St. Louis, MO, USA) dissolved in sterile saline (0.9% w/v NaCl). For a volume of 10 mL weight 0.25g of BrdU in an analytical balance and add it to 10mL of sterile saline solution. For mice, prepare a solution of 5mg/mL and inject a volume equivalent to ten

times the mice weight. For 20 mL of solution weight 0.1g of BrdU in an analytical balance and add it to 20mL of sterile saline solution. In order to easily dissolve the BrdU in saline, warm up the solution while mixing it (*see* **Note 1**). Aliquot the BrdU solution and store it at -20ºC (*see* **Note 2**).

2.2 Immunohistochemistry solutions

1. 4% paraformaldehyde (PFA) in phosphate saline buffer (PBS): 40 g of PFA in 1L of 0.01M PBS. The PFA is dissolved by warming up and mixing the solution in a magnetic stirrer (*see* **Note 3**). Store at 4ºC.
2. Tris-buffer saline (TBS) 0.05M: 6 g Tris base, 9 g NaCl in 1L of dH_2O. Bring to pH to 7.6. Store at 4ºC.
3. TBS-0.2%T: dissolve 2mL of TritonX-100 in 1L of TBS. Store at 4ºC.
5. HCl 2M: add 66mL of HCl 37% to dH_2O and make up to 1L. Store at 4ºC (*see* **Note 4**).
4. Citrate buffer 10 mM: dilute 100x in dH_2O from stock solution citrate buffer 1M (Thermo Scientific, Waltham, Massachusetts, USA)
5. Hydrogen peroxide (H_2O_2) 3%: dilute 10x in TBS from stock solution (H_2O_2 30%)
6. Bovine serum albumin 4% (BSA) (Sigma): 4g of BSA in 100 mL of TBS
7. Tris-HCl 0.05M: 6 g Tris base in 1L of dH_2O, bring to pH 7.6. Store at 4ºC.
8. 3,3'-diaminobenzidine tetrahydrochloride hydrate (DAB) (Sigma) substrate solution, 0.025% (w/v) of DAB in 0.15% (v/v) of dH_2O_2: 75 mg of DAB, 1,5 mL of H_2O_2 30% in 300 mL of Tris-HCl (*see* **Note 5**).
9. Harris Hematoxylin solution (Merck, Frankfurt, Germany), Ammonia solution 25% (Merck) (dilute 100x in dH_2O to 0.25%), ethanol 50, 70, 96, 100%, 100% and Xylenes (Sigma) and Entellan New mounting medium (Merck).

2.3 Immunohistochemistry antibodies

1. Mouse anti-BrdU (DAKO, Clone Bu20a, DAKO, Spain)
2. Rabbit anti-Ki67 (Ki67 antigen, rabbit polyclonal antibody, Novocastra, UK).
3. Secondary antibody: biotinylated goat Anti-Polyvalent (mouse and rabbit) and streptavidin peroxidase from UltraVision detection system (Thermo Scientific)

3. Methods

3.1 BrdU injection

BrdU, an exogenous marker for proliferation, is a thymidine analogue that is incorporated in the DNA during the S phase. Inject BrdU (50mg/Kg) intraperitoneally with a 26 or 25G needle. For rats the volume of injection from the stock solution (25mg/mL) is twice the rat weight, for instance a 300g rat will receive a 600µl injection. For mice, the volume of injection is 10x the weight, i.e., mice with 20g will receive 200µl from the BrdU stock solution (5mg/mL) (*see* **Note 6**).

3.2 Brain freezing procedure and slices collection

1. Upon transcardiac perfusion with saline, collect the brain and place it in a rectangular mold embedded in tissue tek O.C.T. compound (Thermo Scientific) that provides an appropriate matrix for cryostat sectioning at -20°C (*see* **Note 7**).

2. Snap frozen the brain by immersing the mold in a recipient with isopentane and then into liquid nitrogen for a couple of minutes until it is frozen (*see* **Note 8**).
3. Section the brain in a cryostat and collect the sections to Super Frost plus slides (Menzel-Glazer from Thermo Scientific). Make 20µm coronal sections and start collecting all slices as soon as the ventricle begins. Stop collecting brain sections when you see large ventral ventricles at the level of the hippocampus. The bregma coordinates for the beginning and end of the SEZ are the following: rat, bregma coordinates 2.28 mm to -3.60 mm; mice, 1.18mm to -2.06 mm (*see* **Note 9**).
4. The methodology to collect the sections is the following: make series of 8 slides and collect consecutive sections to consecutive slides, i.e., if you have series of 8 slides, in one slide each brain section will be 160µm distant from the subsequent (Figure 1). Following this methodology you will obtain slides with representative sections at a defined constant distance from each other (stereological requirement for proliferation assessment). The sections collected to glass slides should be stored in slide boxes and frozen at -20ºC (*see* **Note 10**).

3.3 Immunohistochemistry for BrdU and Ki67

This procedure is entirely performed at room temperature, unless otherwise indicated.
1. Choose representative slides of the SEZ, i.e., one slide of each series prepared as indicated above (*see* **Note 11**).
2. Fixation: fix the tissue in 4% PFA for 30 min (*see* **Note 12**).
3. Wash 3x 3min in TBS.
4. Permeabilization: incubate the slides 10 min in TBS-0.2%T.
5. Wash 3x 3min in TBS.
6. Antigen retrieval: pre-heat until boiling the 10mM citrate buffer in the microwave. Add the slides to the citrate buffer and leave them immersed at low potency in the microwave for 20 min (*see* **Note 13**).
7. Leave sections to cool down under a hood for approximately 15 min.
8. Immerse rapidly through dH_2O.
9. Acidification (this step is only required for BrdU staining): incubate sections in HCl 2M for 30 min (*see* **Note 14**).
10. Wash 3x 3min and 1x 10min in TBS.
11. Endogenous peroxidases inactivation: incubate sections 10 min in H_2O_2 3% (*see* **Note 15**).
12. Wash 3x 3min in TBS.
13. Block against nonspecific binding: incubate sections in BSA 4% for 30min.
14. Pour the excess of BSA in the slides and place it in a flat humidified chamber. Add the primary antibody (usually 300µl for slide) and incubate overnight at 4ºC. For BrdU (Dako) a dilution of 1:50 in TBS is used, while for Ki67 (Novocastra) the dilution is 1:100 in TBS (*see* **Note 16**).
15. Wash 3x 3min in TBS.
16. Incubate in secondary antibody (Thermo Scientific) for 30min (*see* **Note 17**).
17. Wash 3x 3min in TBS.
18. Incubate in streptavidin peroxidase (Thermo Scientific) for 30min (*see* **Note 18**).
19. Wash 2x 3min in TBS and 1x 3min in Tris-HCl.
20. Develop the reaction in DAB substrate while observing random sections in the microscope (*see* **Note 19**).

21. Counterstain the slides for 5 seconds in hematoxylin for staining tissue nuclei in blue (*see* **Note 20**).
22. Pass through dH_2O and then ammonia solution 0.25%.
23. Dehydrate the tissue through serial passages in increasing alcohol gradients: 3min in ethanol 50, 70, 96 and 100% and finally in xylene (*see* **Note 21**).
24. Using the mounting medium Entellan between them, coverslip the brain sections. Wait 1-2 days until they are dried to start the microscope analysis (*see* **Note 22**).

3.4. Proliferation assessment throughout the SEZ

Sampling methodology: The most important rule in this part is to perform a systematic uniform sampling, i.e., to analyze sections at constant distance intervals, for instance 160µm or 320µm (or even more or less). Using the methodology described above to collect sections in the cryostat, if the analysis if performed for every section in one slide the SEZ brain sections will be separated by 160µm intervals; if the analysis is performed for every other section in a slide, the brain sections will have 320µm intervals between them (*see* **Note 23**).

Random selection of first section: the first section to be analyzed is the one displaying a well-defined juxtaposed ependymal layer (Figure 2) (*see* **Note 24**).

Microscope and software: To estimate the cell proliferation rates throughout the SEZ use, for example, the Visiopharm Integrator system (VIS) software in an Olympus BX51 microscope (Olympus, Hamburg, Germany) or similar software. By using this software you can draw the areas of interest and count within these areas the nuclei stained for BrdU or Ki67 (in brown) with a count tool. Delimitate the SEZ areas at low magnification (40x) and perform the counting of BrdU positive cells within this defined areas at high magnification (400x) (*see* **Note 25**).

Identification of the different SEZ divisions and regions: The coronal sections collected in the cryostat comprise SEZ between bregma coordinates 2.28 mm and -3.60 mm in rat, and 1.18 mm to -2.06 mm for mice (14, 15). Within these coordinates are the anterior, intermediate, posterior and post-posterior SEZ (Figure 3). Table 1 summarizes the divisions of SEZ and the external references used to define it for both rat and mouse. If the analysis is to be performed in different regions of the SEZ in the dorsal-ventral axis, see Figure 3 for detail in area delimitation. Briefly, the anterior SEZ comprises the beginning of the genu of the corpus callosum where a well defined ependymal layer is observed and prolongs to the end of the genu of the corpus callosum. The intermediate SEZ extends up to the decussation of the anterior commissure; the posterior SEZ ends at the beginning of the hippocampus. The post-posterior division of the SEZ is at the level of the hippocampus and finishes with the fusion of the dorsal and ventral parts of the lateral ventricles. From this position on, sparse proliferating cells are detected in the SEZ. Dorsal-ventral axis regionalization comprises dorsal SEZ, located in the upper part of the lateral ventricles; the beginning of the RMS, at the dorsal corner of the lateral wall, the dorsolateral and the ventral SEZ that are the result of the split of the lateral wall in two parts: the ventral SEZ is perpendicular to the corpus callosum, and the dorsolateral SEZ begins at the corner of the lateral wall and extends up to the ventral SEZ where the lateral wall starts to direct to the ventral tip (*see* **Note 26**).

Data processing: The proliferation rates are estimated as the number of BrdU positive cells per area (in mm^2 or μm^2). The data analysis of the SEZ is dependent on the divisions and/or regions assessed. For instance, if the goal is to obtain total proliferation in the intermediate SEZ, determine the rates of proliferation for each section at intermediate SEZ (i.e., the total number of BrdU positive cells divided by the total area) and then average all the sections analyzed for one animal. Repeat this procedure for all animals. Group animals into different experimental conditions and calculate the mean proliferation rate for the group by averaging the proliferation rates of animals within the same group. If, within the intermediate SEZ there is the need to distinguish between regions, i.e., RMS, dorsal, ventral and dorsolateral, proceed as mentioned above. Estimate the BrdU positive cells for each region and the correspondent areas for every section, average rates obtained for each section to obtain the proliferation rate of one animal. Repeat this procedure for all animals and estimate the mean proliferation rate of a determined region for the group. The same rationale is applied to estimate every specific division and/or region of the SEZ (*see* **Note 27**).

Statistical analysis:
Data can be presented as the mean (±SEM) and analyzed with any statistical package software such as GraphPad PRISM 5 software (GraphPad Software Inc., San Diego, CA). The analysis consists of one-way analysis of variance (ANOVA) with Bonferroni multiple comparison post-test analysis for single-factor multiple group comparisons to determine differences between three or more groups.

4. Notes

1. At lower temperatures this solution often precipitates therefore before injecting it to animals confirm that there is no BrdU precipitated. If there is, slightly warm up the solution and dissolve it again.
2. Avoid freezing and thawing, preferably use always fresh. After thawing you will have to resuspend the precipitate by warming up the solution.
3. Prepare this solution one day in advance, it will take some time to dissolve the PFA and afterwards it has to cool down.
4. Preferably use it freshly prepared.
5. Use lab coat, mask and gloves when preparing and using DAB solution, since DAB is carcinogenic. Wrap the solution in aluminium foil to protect from light. Prepare just prior to usage.
6. Animals should be handled for 1 week before the injections in order to minimize the stress-induced changes in the hypothalamus-pituitary axis.
7. Place the brains in the mold oriented in such a position that later it will be ready to glue in the cryostat holder to make coronal sections. The orientation of the brain in the cryostat is crucial to have perfect coronal sections. If the sections are not strictly cut always in the same orientation it can result in divergent areas for the same SEZ position, being assessed between different animals.
8. Isopentane is highly volatile and harmful; this step must be performed in the hood. The time spent in isopentane should be optimized according to the size of the brain, i.e. rat and mice brains take different times to be completely frozen. Store the brains at -20ºC until sectioning.

9. If you are not certain where the ventricles begin, to be on the safe side you should start collecting before you see the ventricles, for instance as soon as you identify the corpus callosum. Use the Rat or Mouse Atlas from Paxinos (14, 15) to identify the main structures of the brain while sectioning.
10. The immunohistochemistry should be performed shortly after the sectioning. Long periods of storage lead to tissue damage and antigen loss. These sections are not pre-fixed in PFA and therefore are more susceptible to degradation.
11. If you choose slide number one of series 1, you should choose slide number 1 of series 2, therefore sections are apart from each other at a constant distance.
12. Since PFA is harmful this step should be performed under the hood.
13. Place the slides into a slide holder and then dive it into a plastic recipient, suitable for microwave, filled with citrate buffer. After 10 min in the microwave, observe if the tissue is not damaged or detaching from the slides during this procedure (this happens if the temperature is too high or if the glass slides used are not appropriate - glass slides should be of the superfrost type).
14. This step allows the linearization of the DNA strands were the BrdU is inserted. If antigen retrieval is not performed prior to this step, 30 min in HCl is not enough to detect BrdU staining; instead 1h in HCl will work, however it may result in nuclear damage, which will difficult the analysis under the microscope. Use fresh HCl 2M and always wear gloves.
15. This step is required to avoid nonspecific staining when developing the immunohistochemistry, because external horseradish peroxidase (HPR) coupled to streptavidin is added to the tissue and will bind to the biotinylated secondary antibody.
16. Verify if 1) the chamber is humid so your antibody solution won't evaporate and 2) the slides are not leaning and therefore the antibody is equally distributed.
17. This antibody can be reused once.
18. Streptavidin can be reused once.
19. Observing in the microscope while development occurs will allow determining the time necessary to see strong brown staining without background, it may vary between 2 to 10 min.
20. Hematoxylin diluted 4x provides a weaker staining and makes it easier to observe the BrdU nuclear brown staining.
21. The slides can be kept in xylene for some minutes until sections are covered with a coverslip.
22. Be careful not to introduce air bubbles between coverslip and sections.
23. Notice that shorter distance intervals will provide you more accurate estimation but will increase the time you will spend on the analysis. Intervals of 160µm or 320µm between analyzed sections typically provide accurate estimations for SEZ proliferation analysis.
24. The first section analyzed must be assigned randomly (stereology principle). In fact the process is already random because it is not known which slide has the first section comprising the beginning of the SEZ. This would only be possible if every section collected would be stained prior to the selection of slides. If you perfuse animals with PFA (and not only saline as described herein) the first section is likely to have the ependymal layer not juxtaposed but instead a slightly opened ventricle can be observed. Furthermore, the areas estimated for the SEZ will be inferior due to the shrinkage of the brain caused by PFA perfusion and by the histological procedures.
25. Alternatively, if you perform all the protocol with fluorescence immunohistochemistry you can do the same analysis by taking images, in a fluorescence microscope or confocal microscope, of the entire SEZ and then estimate the areas and the cell counting numbers in the image J software or in the software provided by the confocal manufacturer.

26: If proliferation is not to be assessed in the entire SEZ, the division(s) of interest to analyze can be selected by following the criteria in Table 1. Most studies on the SEZ focus on the intermediate and the posterior SEZ.

27. To estimate not only the total proliferation but also the proliferation rates in the different regions draw the areas of interest and count the number of BrdU positive cells within those areas, independently. Then, to obtain total area and total BrdU cells, sum the values for all regions, i.e., total=RMS + dorsal + dorsolateral + ventral.

Table 1: Anterior-posterior axis anatomical references for the mouse and rat SEZ divisions. Bregma coordinates are according to Paxinos & Franklin (2001) for mice and Paxinos & Watson (2004) for rat.

SEZ	Bregma coordinates Mouse (mm)	Bregma coordinates Rat (mm)	Anatomical references
Anterior	[1.18; 0.74[	[2.28; 1.44[	From the beginning to the end of the genu of the corpus callosum
Intermediate	[0.74; -0.14[	[1.44; -0.12[	From the end of genu of the corpus callosum to the decussation of anterior commissure
Posterior	[-0.14; -0.94[	[-0.12; -1.72[	From the decussation of anterior commissure to the beginning of the hippocampus
Post Posterior	[-0.94; -1.94]	[-1.70; -3.60]	From the beginning of the hippocampus to the fusion of the dorsal and ventral parts of the lateral ventricle

Figure Legends

Figure 1: Schematic representation of methodology used to collect brain sections. Sections are represented in numbers (from 1 to 40 and then the following consecutive numbers) and are collected following the number order represented in the figure; thus, 1 is the first brain section collected and 41 represents the 41st brain section sliced. The distance between consecutive brain sections collected in the same slide (marked with an * in slide 1 of series 1) is 160µm (20 µm x 8); marked with a + in slides 1 to 8 of series 1 are slices collected consecutively. Because they contain contiguous brain sections, the first slide (1) and the second slide (2) of the first (S1) series are basically identical. To select representative sections of the SEZ choose the necessary series of the same slide number [Sn= series n ($n \geq 1$) of the slide n ($n1 \leq n \leq 8$)].

Figure 2: Detail of the anterior subependymal zone displaying the ependymal layer juxtaposed (arrows) and with BrdU positive cells stained in brown.

Figure 3: Representation of the subependymal zone divisions defined at the anterior-posterior and dorsal-ventral axes. In the upper panel four anterior to posterior divisions are defined according to the SEZ anatomical heterogeneity along the neuraxis: anterior (A), intermediate (I), posterior (P) and post-posterior (PP). For the established divisions, regions are further defined in a dorsal to ventral SEZ orientation, as outlined in the colored traces (middle panel): rostral migratory stream (RMS; red trace), dorsal (blue trace), dorsolateral (orange trace), and ventral (green trace). In the anterior division of the SEZ, the area containing proliferating cells that cannot be defined as RMS is designated undefined (black trace). In the post-posterior division of the SEZ, few proliferating cells are found lining the ventricle wall and therefore no dorsal-ventral region is defined (ventricle walls outlined in grey). The images are from the rat brain. ac, anterior commissure; aca, anterior commissure, anterior part; cc, corpus callosum; DG, dentate gyrus; LV, lateral ventricle. This figure is adapted from (1) under a CC license.

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Figure 1

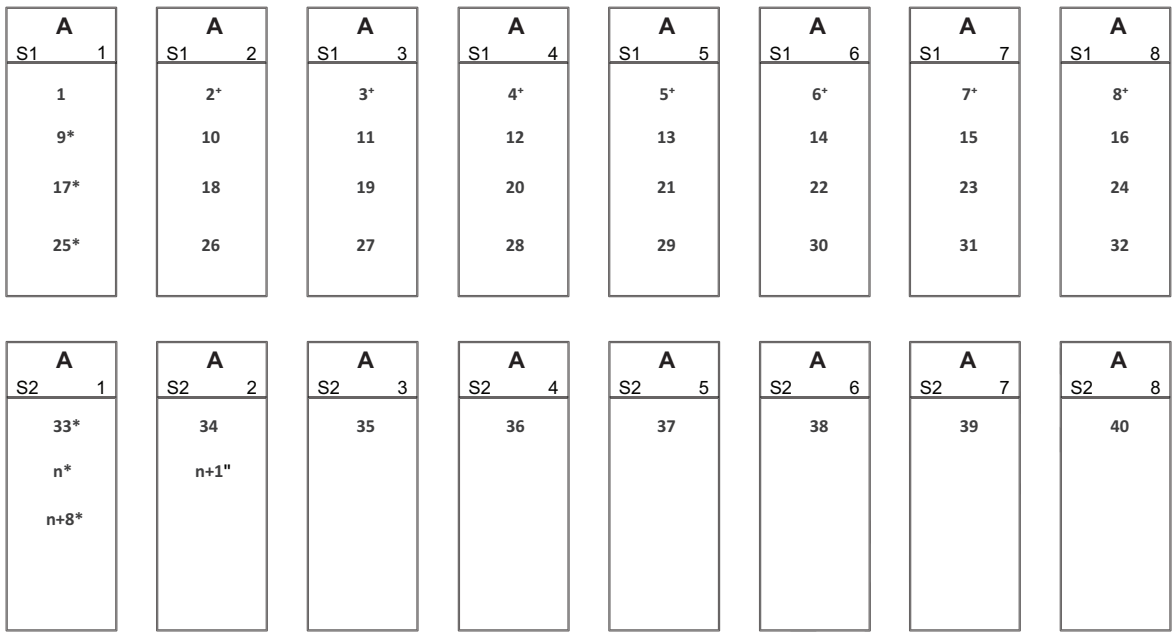

Figure 2

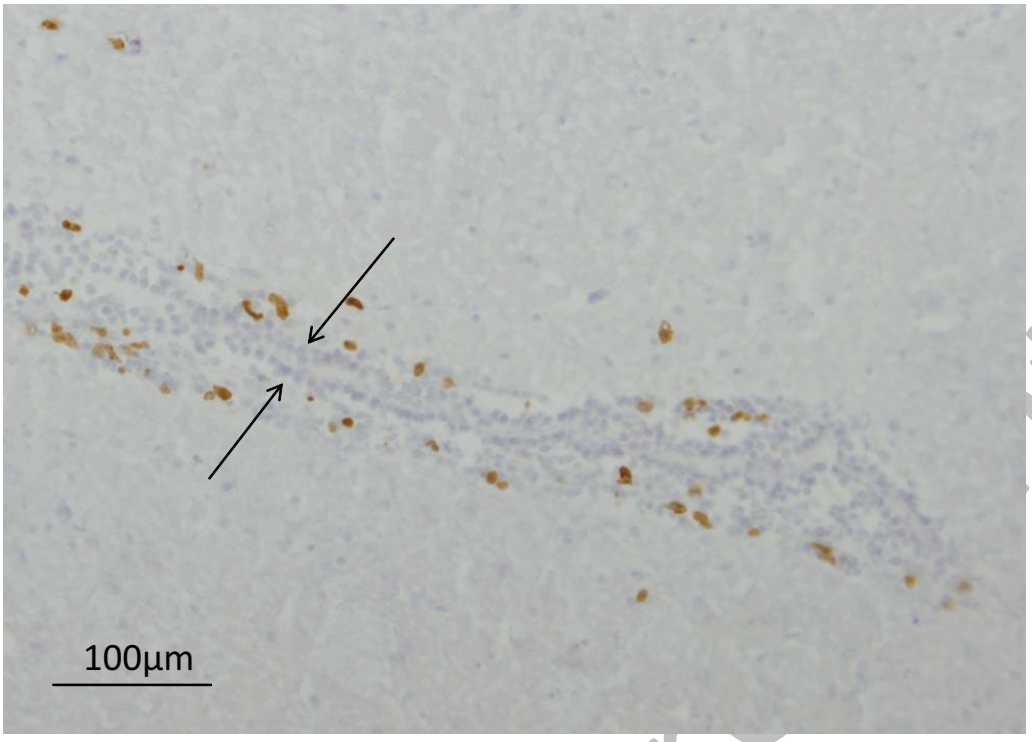

Figure 3