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Immunohistochemistry Techniques to Analyze Cellular Proliferation and Neurogenesis in Rats Using the Thymidine Analog BrdU

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Title of Article: Reviewing different immunohistochemistry methods for studying cellular proliferation and neurogenesis using the thymidine analog BrdU

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Vineeta Bajaj, Ph.D.
Senior Review Editor
Journal of Visualized Experiments

16 July 2020

Dear Dr. Vineeta Bajaj,

Subject: Immunohistochemistry Techniques to Analyze Cellular Proliferation and Neurogenesis Using the Thymidine Analog BrdU. Manuscript No. JoVE61483R1.

Thank you for your email, enclosing the editors and reviewers' comments. We have carefully reviewed the comments and have revised the manuscript accordingly. Our responses are given in a point-by-point manner below. The changes to the manuscript have been tracked to identify all of the manuscript edits. The corrections and suggestions provided by you and the reviewers helped improve the paper. We hope the revised version is now suitable for publication, and we look forward to hearing from you in due course.

Sincerely,
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General Statements about the Revised Manuscript

We strived to cover most of the reviewers' comments. We addressed those comments that we felt were the most relevant and those who stuck to our article's focus. In other cases, we justify the reasons for noncompliance in the Point-by-Point Response to Comments section of this letter (see below). We also performed minor modifications throughout the article adds to those kindly recommended by reviewers to improve clarity and accuracy. Changes in the manuscript were agreed on by all authors. An academic English editor service was hired to revise and proofread the manuscript. All the changes in the manuscript by both the editor and us were tracked. You will find below a point-by-point response to yours' comments. Regular font style is used for featured comments, and italics have been used to show our responses.

TITLE:

Immunohistochemistry Techniques to Analyze Cellular Proliferation and Neurogenesis in Rats Using the Thymidine Analog BrdU

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KEYWORDS:

BrdU, hippocampus, dentate gyrus, neurogenesis, antigen retrieval, detection methods, fixation, immunohistochemistry, immunofluorescence, standardization, troubleshooting, image deconvolution

SUMMARY:

This paper presents four of the most common techniques for visualizing BrdU positive cells to measure adult neurogenesis in rats. This work includes instructions for reagent preparation, thymidine analog administration, transcardial perfusion, tissue preparation, peroxidase immunohistochemical reaction, immunofluorescence, signal amplification, counterstaining, microscopy imaging, and cell analysis.

ABSTRACT:

One of the most important things in the field of adult hippocampal neurogenesis (AHN) is the identification of the newly generated cells. The immunodetection of thymidine analogs (such as 5-Bromo-2'-deoxyuridine (BrdU)) is a standard technique used for visualizing these newly

generated cells. Therefore, BrdU is usually injected in small animals intraperitoneally, so the thymidine analog gets incorporated into dividing cells during DNA synthesis. Detection is performed by immunohistochemical analysis of brain slices. Every research group that has been using this technique can appreciate that it requires special attention to minute details to achieve a successful stain. For instance, an important step is to denture the DNA with HCl. However, the existing scientific reports describe very few of such steps in detail. Therefore, standardizing the technique is challenging for new laboratories as it can take several months to yield positive and successful outcomes. The purpose of this work is to describe and elaborate the steps to obtain positive and successful outcomes of the immunostaining technique in detail when working with the thymidine analog BrdU. The protocol includes the reagent preparation and setup, administration of thymidine analog in a rodent, transcardial perfusion, tissue preparation, peroxidase immunohistochemical reaction, use of avidin-biotin complex, immunofluorescence, counterstaining, microscopy imaging, and cell analysis.

INTRODUCTION:

The idea that new neurons are generated in the adult human brain throughout the lifespan has fascinated the scientific community for decades. The knowledge that the brain generates new neurons throughout its lifespan was attained through the detection of cells under division[1,2] . Detection of newly generated neurons in the adult brain was first identified by intracranially injecting tritiated thymidine (thymidine-H3) in rats and detecting cells in the cell cycle by autoradiograms[1,2]. Cell division of glia and the presence of neuroblasts was reported, which was the first promising data on the postnatal neurogenesis[1]. Nevertheless, the use and detection of thymidine-H3 implied the use of radioactivity, which can be harmful to the people who manage it. The first effort examining the suitability of BrdU immunohistochemistry in the study of proliferation, migration, and origin of cells in the nervous systems appeared in 1988 by Miller and Nowakowski[3]. In 1998, a paper published by Eriksson and colleagues showed that new neurons were visualized postmortem in the human adult brain of patients injected with 5-Bromo-2'-deoxyuridine (BrdU)[4]. These patients received the BrdU injection (250 mg intravenous) to label the growth of tumors[4]. This technique was adopted into animal models. The introduction of these methods marked a milestone for the field since this allowed detection of newly generated cells without the use of radioactive compounds. This procedure became the gold standard to measure cell proliferation in adult brain niches to promote further research in the field.

The limitation of the thymidine analog technique is that it does not allow the determination of cellular identity for the newly generated cells. However, immunohistochemistry allows us to carry out double- or triple- labeling technique of the same cell, which validates the cellular fate of the newly generated cells and even their stages of maturation, leading to further evolution of the field. This method was characterized to differentiate newly generated cells into glia, undifferentiated neurons, or a fully mature granular cell, and even to determine if they are participating actively in the circuitry. Another breakthrough in the field was the use of transgenic models to identify undifferentiated cells under the domain of nestin. The nestin-GFP transgenic mice express an enhanced green fluorescent protein (GFP), which is under the control of the nestin promoter. Nestin is an intermediate filament characterized by progenitor cells[5]. The nestin-GFP transgenic mice allowed to establish early developmental steps involved in

neurogenesis[6]. However, a significant limitation is to be able to maintain a nestin-GFP transgenic mice colony under special conditions in a laboratory facility that becomes cost-effective for some scientific groups, especially those from developing countries.

The techniques mentioned above have advantages and disadvantages. However, identification of proliferating cells by immunohistochemistry (IHC) and the possibility to carry out double- or triple- labeling technique by immunofluorescence to identify cell maturation stage or cell fate represents the most feasible way to measure adult neurogenesis, so far. The identification process using immunohistochemistry consists of labeling proteins, protein domain, or nucleotides with a specific antibody that allows their recognition known as primary antibody. The latter is recognized by the secondary antibody, which is marked with a chromogen (e.g., horseradish peroxidase) or a fluorochrome (e.g., FITC) coupled with the secondary antibody. Microscopes can detect both chromogens and fluorochromes signals. Using IHC, it is possible to identify membrane proteins, cytoskeleton proteins, or nuclear components such as BrdU. On the other hand, BrdU can be found in the cellular nucleus since it is incorporated into the DNA during S-phase by competition. Therefore, a crucial step is the DNA denaturation with HCl, which opens DNA bonds to allow the BrdU antibody access to BrdU within the DNA. It is essential to know that BrdU is present in a saturated concentration in mice and rat serum for 15 and 60 min respectively, after intraperitoneal administration, then drops rapidly to undetectable levels at 60 and 120 min respectively[7].

Here, we describe four different but closely related IHC techniques: chromogenic indirect detection using horseradish peroxidase (HRP) reaction with DAB (3,3'-diaminobenzidine) sans signal amplification (step 4.1), avidin-biotin complex (ABC) amplification (step 4.1), indirect immunofluorescence detection without signal amplification (step 4.4) and labeled streptavidin-biotin (LSAB) amplification (step 4.3). Each method has advantages and disadvantages and could be useful for specific tissue requirements (see **Table 1**). We decided to follow indirect ICH methods due to their affordability and simplicity to make changes from chromogenic to fluorescent detection methods when using unconjugated primary antibodies. The HRP approach is a commonly used IHC method due to its affordability, high stability, high turnover rate, and substrates' full availability. Nevertheless, we recommend using a positive control to confirm that the staining method works accurately and the use of negative control to test the antibody function effectively. Multiple immunostainings or multiplex IHC methods (see step 6) are potent tools to acquire large amounts of data from the tissue section in a single experiment. This technique is particularly important when the availability of samples is limited. Another advantage is the possibility to simultaneously identify specific proteins co-expressed in the same cellular space while preserving tissue integrity. Multiplex allows to stain different markers expressed during specific proliferative stages (e.g., nestin, GFAP, DCX, Ki-67), enabling us to reach a more detailed proliferation and differentiation research[8]. It is crucial to choose antibodies compatible with the fixation technique used to avoid cross-reactivity. We recommend testing each new antibody (including BrdU) individually to adjust and refine the method. Then, introduce the double sequential staining and, finally, start the simultaneous immunostaining process when the sequential method is entirely dominated. It is crucial to choose appropriate secondary antibodies for this method.

[Place **Table 1** here]

A high-resolution image is fundamental to perform proper analysis and present the outcomes. There are two approaches to improve the resolution: 1) use of a better microscope design (e.g., confocal, multiphoton) or 2) numerically inverting the blurring process to enhance images using deconvolution[9]. Unfortunately, confocal microscopy is not affordable due to the high costs of equipment and its servicing[10]. A wide-field epifluorescence microscope and the subsequent deconvolution of the z-stack images provide a suitable, low-cost alternative to confocal microscopy[8,9]. As noted above, deconvolution goal is to restore the original signal that was degraded by the acquisition system[9], by reducing blur, out-of-focus haze and distortion shown in the image obtained by an epifluorescence or confocal microscope using mathematical removal algorithms[10]. The acquired blurred image can be mathematically modeled as the result of convolving the observed objects with a 3D point-spread function (PSF). PSF is a theoretical diffraction pattern of the points of light emitted by the tissue sample and collected by the microscope. PSF file is created with the specific conditions of each image, such as the CCD cell spacing of the camera, refractive index of the media used, the numerical aperture of the objective lens, the emission wavelength of the fluorophore, image sizes, number of images in the z-stack processing method and the space between them (see technical specification in **Table 2**). In other words, the PSF file summarizes the effects of the imaging setup on the microscope observations[9]. However, we use the diffraction PSF 3D Plugin (https://imagej.net/Diffraction_PSF_3D) to create our own specific PSF file for each z-stack image. Z-stack images are a series of digitized optical sections from defined depths (z-axis) at the same XY location of the slide. A computer compiles the information obtained from the focus plane by reassigning signals which have originated from objects located in other focal planes. To create z-stack images, it is necessary to take images from different focused layers of the slides (e.g., ten different images of the same XY area every 1 µm depth). Then, we use microscopy software provided by the manufacturer or Fiji to create a z-stack or 3D image. The result will be a single stack image file (e.g., ten images with different focuses). There are several customer-specific tools and software solutions, such as open-source software for deconvolution microscopy. We will show the outputs of the deconvolution process using DeconvolutionLab2[9] which is a Fiji[11] plugin (distribution of ImageJ[12]). Deconvolution will help improve the resolution of final micrographs (see **Figure 1B,C**). For further information and instruction, we strongly recommend reading reference[13].

[Place **Figure 1** here]

The purpose of this work is to provide a detailed description of the steps to obtain positive and successful outcomes with immunostaining and to list commonly used steps in BrdU-based studies, without the use of a confocal microscope. BrdU staining is a technique that requires several steps that must be carefully followed to achieve a successful stain. Standardizing these staining techniques typically takes months and is time and resource intensive. We anticipated that this article could provide information to the groups starting out within this field by reducing time and errors.

PROTOCOL:

All procedures follow the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications N°. 8023, revised in 1978) and local Mexican laws to minimize the number of animals used and their suffering. The Ethics Committee of the Universidad Iberoamericana approved the experimental protocols for using animals in this study.

1. Reagent preparation and setup

NOTE: Most of the solutions can be prepared days before use unless specified otherwise.

1.1. BrdU solution

1.1.1. Retrieve the BrdU solution from -20 °C freezer and allow it to equilibrate at room temperature (RT).

1.1.2. Calculate the mass of BrdU needed for a dose of 50 mg/kg according to the body weight of the rat. Calculate volume of 0.9% saline solution (0.9 g NaCl in 100 mL of sterile H_2O) needed for a working solution of 20 mg/mL. Prepare an excess to provide at least 0.5 mL per rat per injection.

NOTE: The dose administered to experimental animals should be safe, with minimal side effects, and effective. It has been reported that duration of staining with 100 mg/kg BrdU does not outweigh the potentially higher toxicity compared with the 50 mg/kg dose[7]. No significant differences were found in the number of BrdU-labeled cells/mm^3 for 50 and 100 mg/kg i.p. in rats[7]. It is preferable to inject a small dose to minimize the suffering of the animals.

1.1.3. Weigh out BrdU solution and add it to the saline solution in a conical tube and vortex.

NOTE: Pre-heat the saline solution at 45–50 °C in a water bath for volumes bigger than 1 mL.

1.1.4. Place the tube in a water bath at 50 °C for 10–15 min and vortex every 2–3 min until completely dissolved. Filter the solution with a syringe filter for sterile injection. Cover the tube with tin foil, cool it down at room temperature and use immediately.

CAUTION: BrdU solution is toxic and potentially carcinogenic. Prepare it in the fume hood. BrdU solution must be handled with proper protective equipment (PPE). It is recommended to prepare the solution immediately before use. However, the solution is stable for 24 h under RT. Please protect it from light.

1.2. To prepare 1 L of 0.1 M phosphate buffered saline (PBS) at pH 7.4, add 240 mg of potassium phosphate monobasic (KH_2PO_4), 1.44 g of sodium phosphate dibasic (Na_2HPO_4), 200 mg of potassium chloride (KCl), and 8 g of sodium chloride (NaCl) to 800 mL of double distilled water (ddH_2O) under constant stirring. Adjust the pH to 7.4 and add double distilled H_2O up to total volume of 1 L. Store at 4 °C for up to 1 week.

1.3. For 100 mL of PBS+, add 3% (3 mL) of normal horse serum and 0.3% (300 µL) of Triton X-100 to 0.1 M PBS (pH 7.4). Store in 20–50 mL aliquots at -20 °C for up to 3 months.

NOTE: Alternatively, TBS can be used instead of PBS. Any other serum different from the host's antibodies and experimental tissue is suitable.

1.4. For 100 mL of PBS++, add 10% (10 mL) of normal horse serum and 0.3% (300 µL) of Triton X-100 to 0.1 M PBS pH 7.4. Store in 20–50 mL aliquots at -20 °C for up to 3 months.

1.5. For 1 L of cryoprotectant solution, mix 250 mL of ethylene glycol and 250 mL of glycerol, constantly stir until mixed. Slowly bring to 1 L with PBS. Filter with grade 4 (20–25 µm) filter paper. Store at 4 °C or RT for up to 1 year.

1.6. Prepare 4% paraformaldehyde in 0.1 M PBS (PFA solution) as follows. For 1 L of solution, add 40 g of paraformaldehyde powder slowly to 800 mL of 60–65 °C 0.1 M PBS under constant stirring. Stir until paraformaldehyde is completely dissolved while controlling the temperature (60–65 °C). If necessary, add a few drops of 1 M NaOH to clarify the solution. When the solution reaches room temperature, filter with grade 4 (20-25 µm) filter paper.

CAUTION: Paraformaldehyde is toxic and is suspected of being a carcinogen, prepare in the fume hood. Store at 4 °C for and preferably use within to 2 days. PFA ready-to-use solution is commercially available.

1.7. For 1 L of 10 mM Sodium citrate buffer (SCB) at pH 6, add 1.204 g of sodium citrate (dihydrate), and 1.134 g of citric acid to 800 mL of double distilled H_2O under constant stirring. Adjust the pH to 6.0 and add ddH_2O up to 1 L. Store at 4 °C for up to 6 months.

1.8. Prepare 50 mL of 2 N HCl by slowly adding 8.25 mL of 12 N HCl (concentrated stock solution) to 41.75 mL of ddH_2O under constant stirring.

CAUTION: Prepare in the fume hood. Solution must be prepared immediately before use.

NOTE: 2 N HCl will be used for DNA denaturalization, a crucial step. As BrdU is incorporated into the DNA, HCl is used to open the DNA bonds allowing BrdU antibody access BrdU within the DNA.

1.9. Prepare endogenous peroxidase blocking solution as follows. Prepare 100 mL of 0.6% hydrogen peroxide by mixing 2 mL of 30% hydrogen peroxide with 98 mL of ddH_2O under constant stirring.

NOTE: The solution must be prepared immediately before use. Keep it in the dark as H_2O_2 is light sensitive. PBS or TBS can be used instead of water.

1.10. Prepare avidin-biotin complex (ABC) solution as per the instructions from the manufacturer. For 5 mL of ABC in 0.1 M PBS, add 2 drops (≈100 µL) of reagent A and mix, and then add 2 drops (≈100 µL) of reagent B and mix.

NOTE: Solution must be prepared and allowed to tumble-roll for 20–30 min before use.

1.11. Prepare DAB (Diaminobenzidine) Peroxidase (HRP) substrate using the kit by following the instructions of the manufacturer. To 5 mL of ddH_2O, add 2 drops (≈ 84 µL) of reagent 1 and mix, add 4 drops (≈ 100 µL) of reagent 2 and mix, then add 2 drops (≈ 80 µL) of reagent 3 and mix. Finally, if desired, add 2 drops (≈ 80 µL) of reagent 4 (Nickel) and mix.

NOTE: Solution must be prepared immediately before use.

CAUTION: DAB is toxic and potentially carcinogenic. It must be handled with care and discarded as per the hazardous waste regulation at each institution. To inactivate DAB, add several drops of bleach (sodium hypochlorite); the solution will turn black.

1.12. Prepare 100 mL of cresyl violet solution by adding 100 mg of cresyl violet acetate and 250 µL of acetic acid to 80 mL of ddH_2O at 55–60 °C. Adjust the volume to 100 mL, filter, and store at 4 °C in a dark-colored vessel.

NOTE: The user is encouraged to test different concentrations of the cresyl violet solution before using it on valuable tissue samples. The result may be darker for counterstaining with some tissue samples, which may decrease the ability to count BrdU positive cells accurately.

2. Thymidine analog BrdU administration

2.1. Restrain the experimental animal (e.g., 90-day-old male Wistar rat weighing 350 g), by immobilizing the lower abdominal cavity.

2.2. Administer the BrdU solution (50 mg/kg) intraperitoneally (i.p.) using a 23 G needle and 1 mL syringe.

NOTE: Adjust the injection volume as per weight of the animal. Use a 23–27 G needle and a 1–5 mL syringe for adult rats. The maximum tolerable intraperitoneal injection volume in the adult rat is 10 mL. Different routes can be used to administer the BrdU solution[14]. For example, intraperitoneal injection or oral administration through drinking water.

3. Tissue preparation

NOTE: Three-month-old rats were allowed ad libitum access to physical activity (endless wheel) for seven days. On the day 6, rats were injected with BrdU (step 2) 3 times at intervals of 12 h. Perform steps in section 3 after 8 h from the last BrdU injection.

3.1. Inject pentobarbital (50 mg/kg i.p.) and wait a few minutes until the animal is deeply anesthetized.

NOTE: Make sure that the animal is completely anesthetized before continuing. Carefully pinch one of the legs or the tail. If the animal reacts to the stimulus, wait a few more minutes. If the animal does not react to the pinch, go to the next step.

3.2. Expose the heart by cutting the abdominal cavity skin below the breastbone, taking apart the ribs, and cutting the diaphragm.

3.3. Transcardial perfusion fixation

3.3.1. Insert a needle into the left ventricle and make a small incision in the right atrium. Using a pump or gravity, perfuse (flow rate 5–7 mL/min.) 0.1 M PBS until entire blood is drained out, and the solution becomes clear.

3.3.2. Using a pump or gravity, perform cold perfusion (flow rate 5–7 mL/min) with PFA solution to fix the tissue until the tail becomes rigid.

NOTE: Usually, a 300 g rat requires around 100–150 mL of the PFA solution. The tissue fixation is optional. Thereby, the brain can be extracted for use in various processes to minimize animal usage in the experiments.

3.4. Dissection and post-fixation

3.4.1. Decapitate the animal, and gently extract the brain from the skull. Immerse the brain into a conical tube containing PFA solution (~40 mL for a 250 mg rat) for 1–2 days at 4 °C.

NOTE: Do not over-fixate (more than 48 h), because this can deplete the tissue staining due to the unavailability of antigens.

3.4.2. Prepare 100 mL of 30% sucrose solution, adding 30 g of sucrose to 70 mL of 0.1 M PBS solution under constant stirring. Add 0.1 M PBS solution to 100 mL. Immerse the brain into a conical tube with a 30% sucrose solution (35 mL) for approximately 1–2 days at 4 °C until the brain sinks to the bottom of the tube.

3.5. Cutting coronal brain sections

NOTE: Using a cryostat-microtome requires guidance and training. For detailed instructions, see reference[15].

3.5.1. Submerge the whole brain into iso-pentane at -80 °C and keep it at -80 °C for 10 min. Place the brain in an embedding matrix on a cryostat-microtome plate.

NOTE: Under certain conditions, rapid freezing of the brain at -80 °C can cause fracture or damage to the tissue. The user should be aware of this problem. If this is the case, use -20 °C iso-pentane to freeze the brain.

3.5.2. Using a cryostat-microtome (temperature at -25 to -20 °C) cut coronal sections of 40 µm thickness. Sequentially transfer sections into a 24-well cell culture plate with cryoprotection solution following the guide in **Figure 2**. Store at -20 °C until use, for up to few months.

NOTE: Hereafter, process all tissue in free-floating serial sections of 40 µm in 12-well plates with mesh inserts in gentle and continuous agitation (10 rpm). It is possible to store brain sections for years under the right conditions.

[Place **Figure 2** here]

4. Immunostaining

NOTE: See **Table 1** for the summary of the advantages and disadvantages of each technique.

4.1. Detection of BrdU using peroxidase reaction with DAB

NOTE: Perform steps 4.1.1 to 4.1.5 on day 1.

4.1.1. Transfer slices from the cryoprotection solution to 0.1 M PBS at room temperature. Rinse three times for 10 min each, with 0.1 M PBS.

4.1.2. Incubate slices for 30 min in endogenous peroxidase blocking solution to inactivate endogenous peroxidase. Rinse 3 times, 10 min each, with 0.1 M PBS. Optionally, perform antigen retrieval (see section 5). Incubate slices for 20 min with 2 N HCl at 37 °C. Rinse in 0.1 M borate buffer (8.5 pH) for 10 min. Rinse 3 times for 10 min each, with ice-cold 0.1 M PBS.

4.1.3. Incubate slices for 2 h at room temperature with PBS++ (blocking solution). Incubate with anti-BrdU primary antibody (mouse host) at concentration of 1:250 in PBS+ overnight at 4 °C.

4.1.4. On day 2, rinse the slices 3 times for 10 min each with 0.1 M PBS.

4.1.5. Incubate with 1:250 HRP-conjugated secondary antibody (anti-mouse) in PBS+ for 2–4 h at room temperature. Rinse 3 times for 10 min each with 0.1 M PBS.

4.1.6. Transfer slices to DAB Peroxidase (HRP) Substrate solution and incubate for 2–10 min. When slices become dark grey, visualize the tissue with a magnifying glass or a microscope. If positive cells are present, rinse 3 times (for 15 min each) with tap water (to reduce background). Wash 3 times for 10 min each with 0.1 M PBS.

4.1.7. Carefully mount slices on gelatinized slides using a soft brush, air dry overnight at room temperature. Counterstain (see section 7.1), add permanent mounting medium and place coverslips. Store at 4 °C for up to 6 months.

4.2. Detection of BrdU using peroxidase reaction with the avidin-biotin-peroxidase complex

NOTE: Perform steps 4.2.1 to 4.2.5 on day 1.

4.2.1. Transfer slices from the cryoprotection solution to 0.1 M PBS to bring to room temperature. Rinse 3 times for 10 min each with 0.1 M PBS.

4.2.2. Incubate for 30 min with endogenous peroxidase blocking solution to inactivate endogenous peroxidase. Rinse 3 times for 10 min each in 0.1 M PBS. Optionally, perform antigen retrieval (see section 5).

4.2.3. Incubate for 20 min with 2 N HCl at 37 °C. Rinse in 0.1 M borate buffer (pH 8.5) for 10 min. Wash 3 times for 10 min each with ice-cold 0.1 M PBS.

4.2.4. Incubate for 2 h at room temperature in PBS++ (blocking solution).

4.2.5. Incubate with anti-BrdU primary antibody (mouse host) 1:250 in PBS+ overnight at 4°C.

4.2.6. On day 2, rinse 3 times for 10 min each with 0.1 M PBS.

4.2.7. Incubate with 1:250 biotinylated secondary antibody (anti-mouse) in PBS+ for 2–4 h at room temperature. Rinse 3 times for 10 min each with 0.1 M PBS.

4.2.8. Incubate in the ABC solution for 1 h at room temperature. Rinse 3 times for 10 min each with 0.1 M PBS.

4.2.9. Transfer slices to DAB peroxidase (HRP) substrate solution and incubate for 2–10 min. When slices become dark grey, visualize the tissue with a magnifying glass or a microscope. If positive cells are present, rinse 3 times (15 min each) with tap water (to reduce background) followed by 3 times with 0.1 M PBS wash for 10 min each.

NOTE: Solution must be prepared immediately before use. Care should be taken to avoid brain slices sticking to each other due to irregular dark spots in the tissue.

4.2.10. Carefully mount slices on gelatinized slides using a soft brush and then air dry overnight at room temperature.

4.2.11. Counterstain if needed (see step 7.1), add permanent mounting medium and place coverslips. Store at 4 °C for up to 6 months.

4.3. Detection of BrdU by immunofluorescence using labeled Streptavidin-Biotin (LSAB) amplification

NOTE: Perform steps 4.3.1 to 4.3.4 on day 1.

4.3.1. Transfer slices from the cryoprotection solution to 0.1 M PBS at room temperature. Rinse 3 times for 10 min each with 0.1 M PBS. Optionally, perform antigen retrieval (see section 5).

4.3.2. Incubate for 20 min in 2 N HCl at 37 °C. Rinse in 0.1 M borate buffer (8.5 pH) for 10 min. Rinse 3 times for 10 min each in ice-cold 0.1 M PBS.

4.3.3. Incubate for 2 h at room temperature in PBS++ (blocking solution). Incubate with 1:250 anti-BrdU primary antibody (mouse host) in PBS+ overnight at 4 °C.

4.3.4. On day 2, rinse 3 times for 10 min each with 0.1 M PBS.

4.3.5. Incubate with 1:250 biotinylated secondary antibody (anti-mouse) in PBS+ for 2–4 h at room temperature. Rinse 3 times for 10 min each with 0.1 M PBS. Incubate with 1:250 fluorochrome-conjugated streptavidin (Cy3) in PBS (do not use serum) for 1–2 h at room temperature. Rinse 3 times for 10 min each with 0.1 M PBS.

NOTE: Serum can contain biotin and should not be added to diluents. Instead, use PBS containing 0.3% of Triton X-100.

4.3.6. Carefully mount slices on gelatinized slides using a soft brush, air dry overnight at room temperature, or mount immediately with an appropriate mounting medium. Counterstain (see step 7.2), add permanent mounting medium and place coverslips. Store at 4 °C for up to 6 months.

4.4. Detection of BrdU by indirect immunofluorescence

NOTE: Perform step 4.4.1 to 4.4.4 on day 1.

4.4.1. Transfer slices from the cryoprotection solution to 0.1 M PBS until it reaches room temperature. Rinse 3 times for 10 min each with 0.1 M PBS. Perform antigen retrieval if required (optional, see section 5).

4.4.2. Incubate for 20 min in 2 N HCl at 37 °C. Rinse in 0.1 M borate buffer (8.5 pH) for 10 min. Rinse 3 times for 10 min each with ice-cold 0.1 M PBS. Incubate for 2 h at room temperature with PBS++ (blocking solution). Incubate with 1:250 anti-BrdU primary antibody (mouse host) in PBS+ overnight at 4 °C.

4.4.3. On day 2, rinse 3 times for 10 min each with 0.1 M PBS.

4.4.4. Incubate with 1:250 fluorochrome-conjugated secondary antibody (anti-mouse) in PBS+ for 2–4 h at room temperature. Rinse 3 times for 10 min each with 0.1 M PBS.

4.4.5. Carefully mount slices on gelatinized slides using a soft brush, air dry overnight at room temperature, or mount immediately with an appropriate mounting medium. Counterstain (see step 7.2), add permanent mounting medium and place coverslips. Store at 4 °C for up to 6 months.

5. Antigen retrieval (optional)

NOTE: Antigen Retrieval is an optional step intended to correct the loss of antigenicity caused by fixation that modifies the tertiary and quaternary structure of many antigens, making them undetectable by antibodies. This step can be added to the original protocol.

5.1. In a microwave or water bath, pre-heat 10 mM sodium citrate buffer (SCB) pH 6 solution to 90–95 °C (depending on altitude, the solution starts boiling around this temperature). Fill 80% of a 50 mL conical tube (40 mL) with pre-heated SCB. Transfer slices to mesh inserts into the conical tube with SCB. Cover the tube with a screw cap with holes made with an 18–20 G needle.

5.2. Keep slices for 30 min in SCB at 80–85 °C alternating warming cycles in the microwave at the minimum power level. If needed, refill the conical tube with SCB. Transfer slices immediately after along with the mesh inserts into ice-cold 0.1 M PBS and rinse 3 times for 10 min each.

6. Multiple immunostainings (optional)

NOTE: See the introduction section for the rationale behind this step.

6.1. Simultaneous multiple immunostainings

6.1.1. Prepare a cocktail with the primary antibodies to the target (e.g., mouse anti-BrdU, and rabbit anti -GFAP) in PBS+. Use different hosts for each primary antibody used. Incubate overnight at 4 °C. Continue with the same next steps for each protocol.

6.1.2. Prepare a cocktail with the corresponding secondary antibodies for each primary antibody used (e.g., goat anti-mouse FITC, goat anti-rabbit TRITC) in the same diluent solution for each protocol. Continue with the same next steps for each protocol. Ideally, use secondary antibodies that come from the same hosts to avoid cross-reaction.

6.2. Sequential multiple immunostainings.

6.2.1. Follow the protocol for the first antibody target (e.g., mouse anti-BrdU) and stop before the mounting the slices. Incubate for 2 h at room temperature with PBS++ (blocking solution).

6.2.2. Incubate the second primary antibody (e.g., rabbit anti -GFAP) in PBS+ overnight at 4 °C. Follow the next steps for each protocol, including the incubation of the second secondary antibody (e.g., goat anti-rabbit TRITC). Continue with the next steps for each protocol to the end.

7. Counterstaining (optional)

7.1. For protocols using peroxidase reaction, pre-heat the cresyl violet solution to 60 °C. Hydrate the slides with ddH_2O for 1 min. Incubate the slides in the hot cresyl violet solution for 5–20 min.

7.1.1. Rinse the slides with ddH_2O for 1 min. Rinse the slides with 70%, 80%, 90% and 100% ethyl alcohol for 1–3 min each. Rinse the slides with xylene for 1–3 min.

7.1.2. Add permanent hydrophobic mounting medium and place coverslips.

NOTE: Store at 4 °C for up to 6 months. Self-made mounting medium containing PVA (Polyvinyl alcohol)-DABCO can be employed.

7.2. For protocols using immunofluorescence, add a small volume (25–50 µL) of hydrophilic mounting medium with DAPI, propidium iodide or similar. Seal around the perimeter with nail polish or a plastic sealant. Store at 4 °C for up to 6 months.

8. Imaging and analysis

NOTE: See **Table 2** for microscope setup specifications. Usually, counting the stained new cells is done using the peroxidase reaction stained slices (cheaper method), but it can also be performed using immunofluorescence.

8.1. To quantify cells, first, identify the dentate gyrus properly with the 4x magnification lens (for further instructions on DG anatomical detail, see Amaral et al.[16]).

8.1.1. Search the granular cell layer of the dentate gyrus for nuclei labeled with BrdU (using the 40x magnification lens). Perform cell search exhaustively along the z-axis since new cells can be distributed in different layers (see **Video 1**).

8.1.2. Select an interval section for cell searching all over the dentate gyrus (e.g., every 6th section of tissue, equivalent to every 240 µm).

8.1.3. Count all BrdU positive cells. The morphology of the labeled nucleus can change depending on how much BrdU the cell incorporated (see **Figure 3** as a guide). Move slowly over the z-axis to quantify all several nuclei that integrate a cluster (see **Video 2**).

8.1.4. Multiply the total number of counted cells with the interval section selected (e.g., 6) to estimate the total number of BrdU-labeled new cells.

8.1.5. Ideally, in a regular experiment, count at least ten sections per animal and at least five animals per group.

8.2. Image deconvolution (optional)

NOTE: Refer to the introduction section for important information about this step. This procedure needs monochromatic images (grayscale). Transform color images to grayscale. If the images are an RGB composite, first split the channels and merge them as a single image (not composite), then transform to 8-bit grayscale.

8.2.1. Create a z-stack file from micrographs.

8.2.2. Create a point spread function (PSF) file opening the Diffraction PSF 3D plugin (https://imagej.net/Diffraction_PSF_3D) from the option **Plugins** menu. Fill out all the required data (see **Table 2**). Press **OK** and save the file.

8.2.3. Open the DeconvolutionLab2[9] plugin from the option **Plugins** menu (http://bigwww.epfl.ch/deconvolution/deconvolutionlab2/). Drag the matched z-stack image and PDF file to the corresponding window slot.

8.2.4. Select the deconvolution algorithm (e.g., Richardson-Lucy) and the number of iterations (e.g., 20). Press **RUN**.

8.2.5. Combine the deconvoluted images into a single z-stack image selecting **Stacks** from the **Image** menu at the top. Then click **Z Project.** Select **Max Intensity** from the **Projection Type** dropdown menu, press **O**K and save the file.

8.2.6. Create an RGB image using the single z-stack image file created in the step above with the desired pseudocolor selecting **Color** from the **Image** menu at the top. Then click on **Merge Channels**. Set the corresponding image to the desire color channel from the dropdown menu. Uncheck the box **Create Composite**, press **OK** and save the file (see **Figure 4**).

8.2.7. If there is more than one channel image, repeat the steps 8.2.1–8.2.5. Create an RGB image file following the step 8.2.6, opening at least two image files and selecting different color channels for each image file (see **Figure 4**).

REPRESENTATIVE RESULTS:

The methods described above were applied to quantify newborn cells in adult rat hippocampus after voluntary physical activity, in contrast with a control group without any extra physical activity. We used the postnatal rat hippocampus as a positive control. Male rats 3 months of age were under a voluntary physical activity protocol (endless wheel) for seven days. On day 6, rats were injected with BrdU (section 2), and every 12 h after until three complete injections. To complete three cell cycle divisions, the animals were transcardially perfused (section 3) 8 h after the last BrdU injection. The same procedure was used on three-month old rats which did not

undergo physical activity to be used as a comparative control. As a positive control, one-day-old rat pups (postnatal day 1) were injected with BrdU one time, as described in section 2 above. One day after the injection (postnatal day 2), pups were euthanized, and their heads were immersed in PFA solution, as described in step 3.4. Adult rats were deeply anesthetized (step 3.1), transcardially perfused, as described in step 3.2. Brains were dissected and post-fixated (step 3.4). Brains were cut into 40 µm coronal sections (step 3.5). Sections were processed for BrdU immunohistochemistry, as described in step 4.

We used horseradish peroxidase reaction with DAB IHC for staining (step 4.1) and counting BrdU-positive cells in DG. **Figure 5** shows a DG section with BrdU-labeled cells. **Figure 5C,D** shows a representative part of the DG section at higher magnification. Labeled cells showed intense dark staining, which were marked with arrows. The inset shows the average numbers of labeled cells in the experimental and control groups (counted positive cells multiplied by six as described in step 8.1). A Student's t-test revealed significance differences between the numbers of BrdU-positive cells ($t_{(10)} = 2.704$, $p = 0.0222$). The control group that did not undergo physical activity showed 2,040 ± 314 cells (n = 6 rats). In comparison, the physical activity group showed, on average, 3,606 ± 486 (n = 6 rats) BrdU-positive cells. As observed, physical activity exposure increases BrdU-positive cells. Therefore, these results are consistent with other reported results that show that physical activity increased cellular proliferation in the adult dentate gyrus[17].

FIGURE AND TABLE LEGENDS:

Figure 1: Representative image of 3D deconvolution for multiple color channels. (**A**) DG at low magnification. (**B**) The original z-stack images for each channel and the merged image. (**C**) 3D deconvoluted z-stack images for each channel and the merged image. This brain was from the rat that was the part of physical activity group. Labeled streptavidin-biotin (LSAB) amplification method was used. It showed Cy3 streptavidin conjugated antibody for indicating BrdU (red), DAPI as a counterstaining (blue), and glial fibrillary acidic protein (GFAP) as an astroglial marker (green). ML = molecular layer; GCL = granular cell layer; SGZ = subgranular zone.

Figure 2: Schematic illustration of sequentially transferring sections from cryostat-microtome into a 24-well cell culture plate with cryoprotection solution. Begin at A1-well and place the next slices into row A; after A6-well move to the next row B, so on. When arriving D6, go back to A1 and continue. This arrangement allows Nth (e.g., sixth for neurogenesis, equivalent to the content of one column) section quantification of an entire brain region.

Figure 3: Examples of different morphology of BrdU-labeled cell nucleus. BrdU is a DNA synthesis marker which labels the nucleus. In the hippocampus region, the BrdU-positive nuclei had a semi-oval shape located in the dentate gyrus sub-granular zone. Since BrdU is incorporated by competition, the amount incorporated for every cell will have a variation that will later be reflected in how the nucleus will be visualized. (**A**) Immunofluorescence image. (**B**) An image using peroxidase reaction without an additional amplification method is presented. Yellow arrows show artifacts and non-specific signals. Black or white arrows show BrdU+ cells. 1 – Fully filed nucleus, semi-oval nuclei highly colored. 2 – Nuclei with dots, the border of the nuclei is

marked and has inside several dots. 3 – Nuclei with few dots, the border of the nuclei are marked and have a small number of dots inside. 4 – Small nucleus is possible cells in a different differentiation stage but still part of the niche. 5 – Clusters are precursor cells under division, therefore several cells together in condensed groups can be observed. Within these groups, the counting must be done especially carefully to avoid mislabeling positive cells. Red arrows show the nucleus under division that can be confused to be a single cell. Each cell is enclosed in a box and can be distinguished in a Z-axis plane in real-time.

Figure 4: Representative RGB image for single and merged channels. The top image shows the original z-stack image, and the lower image shows the 3D deconvoluted z-stack image. (**A**) Low magnification of the DG. (**B**) RGB image for each channel, and (**C**) RGB merged image. This was a brain from the control group. Immunofluorescence was used without an additional amplification method. BrdU (red), DAPI as a counterstaining (blue), and GFAP (glial fibrillary acidic protein) as an astroglial marker (green). ML = molecular layer; GCL = granular cell layer; SGZ = subgranular zone.

Figure 5: Representative DG section with BrdU labeled cells (intense dark) for each experimental group. The peroxidase reaction was used with the avidin-biotin-peroxidase complex amplification method. (**A, B**) Show a low magnification of the DG, and (**C, D**) show the box area at higher magnification. Panels A and C are tissues from the physical activity group, panels B and D are from the control group. The inset shows the average numbers of labeled cells in the physical activity and control groups (counted positive cells multiplied by six as described in step 8.1). ML = molecular layer; GCL = granular cell layer; SGZ = subgranular zone; arrows indicate BrdU+ cells.

Video 1: Video showing a different focus of positive cells along the z-axis distributed in different layers.

Video 2: Video showing a different focus of positive cell clusters along the z-axis distributed in different layers. Move slowly over the z-axis to quantify all several nuclei that integrate a cluster.

Table 1: Advantages/disadvantages of IHC techniques. This table shows the advantages/disadvantages for indirect detection methods: Peroxidase reaction with (3,3'-diaminobenzidine) DAB and fluorescence; and signal amplification methods: avidin-biotin complex (ABC), labeled streptavidin-biotin (LSAB), and not additional amplification method.

Table 2: Microscope setup specifications and point spread function (PSF) file creation requirements. There are 11 slots in the diffraction PSF 3D plugin window to create the PSF file. Each slot is described as follows: *1 - Index of refraction of the media: index of refraction for the medium between the slide and the lens (e.g., air = 1.00029). *2 - Numerical Aperture: NA of lens used (it must be corrected when a different immersion media is used and lens was assigned to). *3 - Wavelength: Fluorochrome maximum emission wavelength (nm). *4 - Longitudinal Spherical Aberration: 0.00. *5 - Image pixel spacing: CCD pixel size (nm)/Magnification (e.g., 3.4 µm and 100X lens, 3400/100 = 34 nm). *6 - Distance between images Z-axis. *7 - Width: Enter the width

of the image to be deconvolved in pixels. *8 - Height: Enter the height of the image to be deconvolved in pixels. *9 - Depth, slices: the number of images in the z-stack. *10 - Normalization: Sum of pixel values = 1. *11 - Title: Desired name for the PSF file. The file should match up with the unique given z-stack image.

DISCUSSION:

Adult neurogenesis is a process that occurs most frequently in niches of adult neural precursor cells that have the potential to generate new neurons throughout their lifespans. Bromodeoxyuridine (BrdU) labeling is widely used in immunology to characterize the number of newly generated cells in an adult brain. BrdU will be mainly incorporated into cells of discrete brain regions (neurogenic zones). These cells are located in the sub-ventricular zone (SVZ), the dentate gyrus of the hippocampus—between the hilus and granular cells know as sub-granular zone (SGZ)[1,2,18]. Moreover, there are different brain regions characterized by a lower proliferative capacity in adulthood, including the hypothalamus, striatum, neocortex, and amygdala[19]. As mentioned before, BrdU staining is the commonly used method for adult neurogenesis research to detect cell proliferation. However, the use of BrdU as a marker has limitations and pitfalls. The first one is that BrdU is a cell cycle marker. Therefore, double or triple staining must be performed to identify the cell fate and include cell markers to detect the specific developmental stage of the cells labeled. One more concern about BrdU is that it is a toxic and mutagenic solution that modifies DNA stability may alter cellular function and cell cycles. Consideration should be given to the previous information when deciding to follow an administration protocol and administration doses (50–600 mg/kg). Another crucial feature is that BrdU is a DNA synthesis marker, not a cell proliferation marker[14]. Therefore, it is relevant to distinguish cell proliferation from other events such as a DNA repair, abortive cell cycle re-entry, and gene duplication. Researchers must follow appropriate controls to ensure the appropriate use of BrdU. For a more detailed discussion about these problems and limitations, we recommend reviewing Taupin's work[14]. The standardization process of an immunohistochemistry protocol could be slow and challenging. In this work, we have presented all the general steps to manage a successful IHC protocol. However, we recommend that every research group test and evaluate tissue, antibodies, and conditions in advance. Tests and evaluations must be carried out with at least three different levels of incubations, washing steps, and strengths for each antibody and tissue tested. We also recommend that researchers review additional protocols to be able to choose the best one that meets specific needs and requirements[20–25].

As mentioned previously, the procedure involves several steps and methodological considerations that are commonly used and mentioned in scientific articles, which will be later discussed. We recommend that researchers choose the antibodies carefully and correctly in terms of technique, budget, equipment, setup, and main research goal. Antibodies must be tested with the same type of tissue that will be later tested in the experiment. We also recommend the use of an antibody that was tested for the same purpose (IHC) (i.e., not just in western blot or flow cytometry techniques) to test its compatibility with the fixation technique. Different routes could be used to administer the BrdU staining such as intraperitoneal injection, intraperitoneal infusion, oral ingestion, or intraventricular infusion (for a more detailed description of each technique, see reference[26]). If the intraperitoneal injection is selected, make

sure that BrdU is administrated into the peritoneal cavity avoiding the intestine area. Since the intestine has several cells in duplication that can exhaust the BrdU before it gets to the brain which will affect the number of labeled cells. It is crucial to obtain thin sections since they allow a better penetration of solutions. Coronal slices of 40 µm thick were cut rostro-caudally and were transferred into a 24-well cell culture plate, following the stereological procedure proposed by Kempermann et al.[27]. The immunohistochemistry can be carried out with tissue mounted on slides or as free-floating sections. Since BrdU is located deep in cells nuclei, it allows the penetration of solutions in free-floating sections which provides better results and better access to the area of interest. It is important to open DNA bonds (DNA denaturalization) to allow the primary anti-BrdU antibody access. In this work, we carried out these specific procedures with the use of HCl incubation. On the other hand, the process of blocking unspecific epitopes allowed a more accurate identification of cell signal.

The good membrane permeabilization allows the antibodies to properly penetrate the interest area. Adding a permeabilizer such as Triton X-100 to PBS++ and PBS+ solutions improves membrane permeabilization. Both PBS and Tris-buffered saline (TBS) reagents can be used in this protocol. In terms of budget, the TBS could be relativity cheaper than the PBS. However, PBS could interfere with anti-phosphate antibodies and inhibit alkaline phosphatase-conjugated antibodies, so avoid the use of PBS if the target is post translationally modified by phosphorylation (i.e., phosphorylated). We used the PBS for this work, and we found out that tissue washing steps gave a more specific signal. We also recommend researchers to carry out at least three washing cycles using either TBS o PBS. The solutions must be freshly prepared. Antigen retrieval (AR) is a method intended to reduce the loss of antigenicity caused by the fixation which modifies the tertiary and quaternary antigens' structure. This reduction makes antigens undetectable by antibodies[28,29]. The heat-induced epitope retrieval (HIER) used in this protocol attempted to reverse the chemical reactions between formaldehyde and proteins by high temperature or strong alkaline hydrolysis (with other buffer solutions as EDTA pH 8.5 or Tris pH 9.5). It is essential to test new antibodies with different AR protocols to compare results and choose the best one for the protocol. This last step might be optional in a regular protocol; however, we treated tissues with an antigen retrieval protocol to provide better results for this protocol.

It is crucial to select the correct final contrasting color and the counterstain technique in consideration of the primary staining color and method used to make a non-staining structure visible and avoid masking the primary staining color from the immune reaction. For the fluorescence microscopy, the DAPI (4', 6-diamidino-2-phenylindole) is a very popular nuclear and chromosome counterstain that emits blue fluorescence (absorption: 360 nm, emission: 460 nm) upon binding to AT regions of DNA. DAPI-containing mounting medium is available and is easy to use; this provides excellent signal retention for image acquisition. For the peroxide reaction, IHC was available in different options such as cresyl violet, hematoxylin, neutral red, or methyl green staining. For multiple immunostaining techniques, it is crucial to choose a compatible antibody with the fixation technique used to avoid cross-reactivity[30]. When issues and complications with single staining are solved, administer another color staining as deemed necessary. It is crucial to control the non-specific binding between the secondary antibodies. This could be done by

saturating the primary antibodies before using a secondary antibody produced in the same host species of the primary antibodies. For example, when using anti-mouse produced in rabbit and anti-rabbit produced in goat secondary antibodies, the anti-rabbit produced in goat antibody must be used before the anti-mouse produced in rabbit antibody. When the sequential method is dominated completely, then the simultaneous immunostaining process can be initiated. In this method, it is essential to choose secondary antibodies appropriately. Ideally, all those antibodies must come from the same host animal to avoid cross-reactivity. We recommend running a positive control to confirm that the staining method works accurately in postnatal hippocampus tissue (abundant neurogenesis around this age). If the positive control tissue shows staining problems, review and go over the procedure, make corrections and adjustments, and repeat until a good staining is produced. Then, run a negative control to test that the antibody works correctly by omitting or replacing a particular primary antibody with normal serum (same species as the primary antibody). As mentioned in the introduction, image deconvolution is a powerful tool and provides an alternative when a confocal microscope is not available. It can apply the image deconvolution to all images gained using transmitted light bright-field, wide-field fluorescence, and confocal fluorescence microscopy. The ultimate purpose of image deconvolution is to reconstruct the original signal that the acquisition system deteriorates[10].

In summary, the identification of the newly generated cells visualized by the immunodetection of thymidine analog BrdU is a complicated but powerful technique. This work is an attempt to help scientists, particularly in the field of adult hippocampal neurogenesis, to quantify new cells more accurately. We hope that this effort has been helpful to the scientific community and makes it easier to fine-tune the study of cell proliferation by the immunohistochemistry technique.

ACKNOWLEDGMENTS:
We would like to thank Mr. Miguel Burgos, and Gustavo Lago for providing technical assistance. We also want to thank Dr. Clorinda Arias, Dr. Karla Hernandez and Dr. Oscar Galicia for their kind support in providing reagents and material. We also thank Dirección de Investigación y Posgrado of the Universidad Iberoamericana Ciudad de México for providing funding for the performance of this work and for covering video production expenses.

DISCLOSURES:
The authors have nothing to disclose.

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FIGURE 1

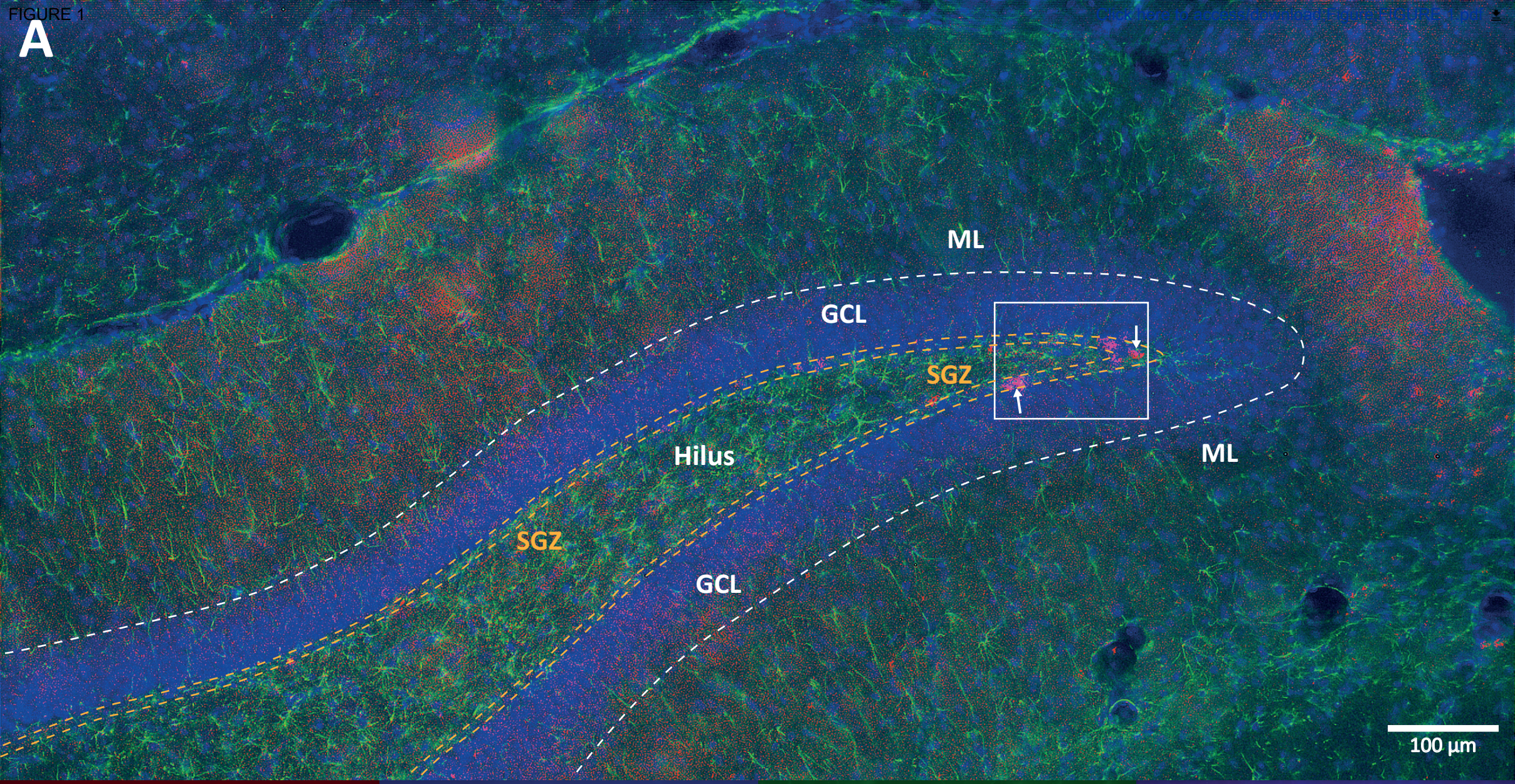
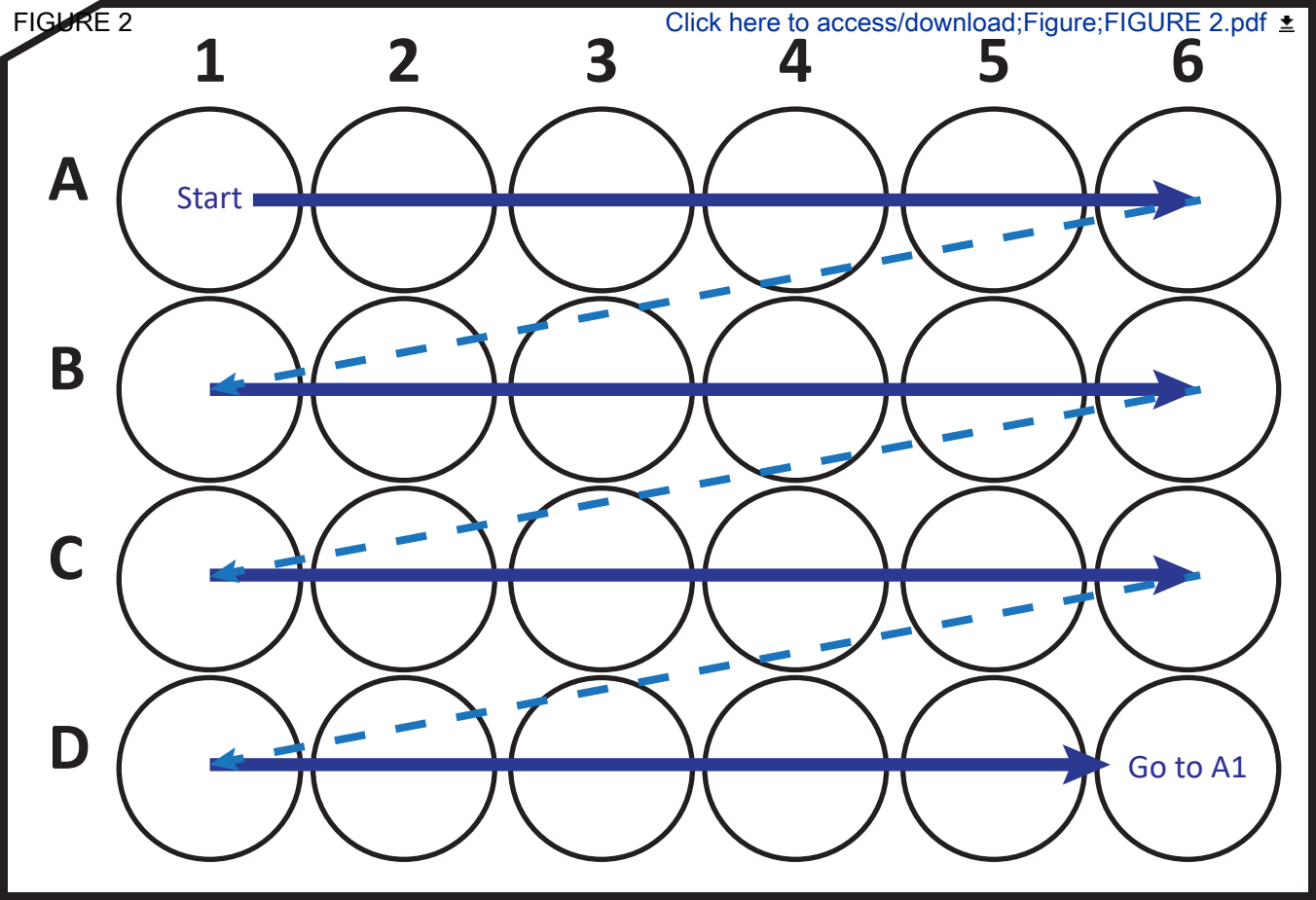


FIGURE 2



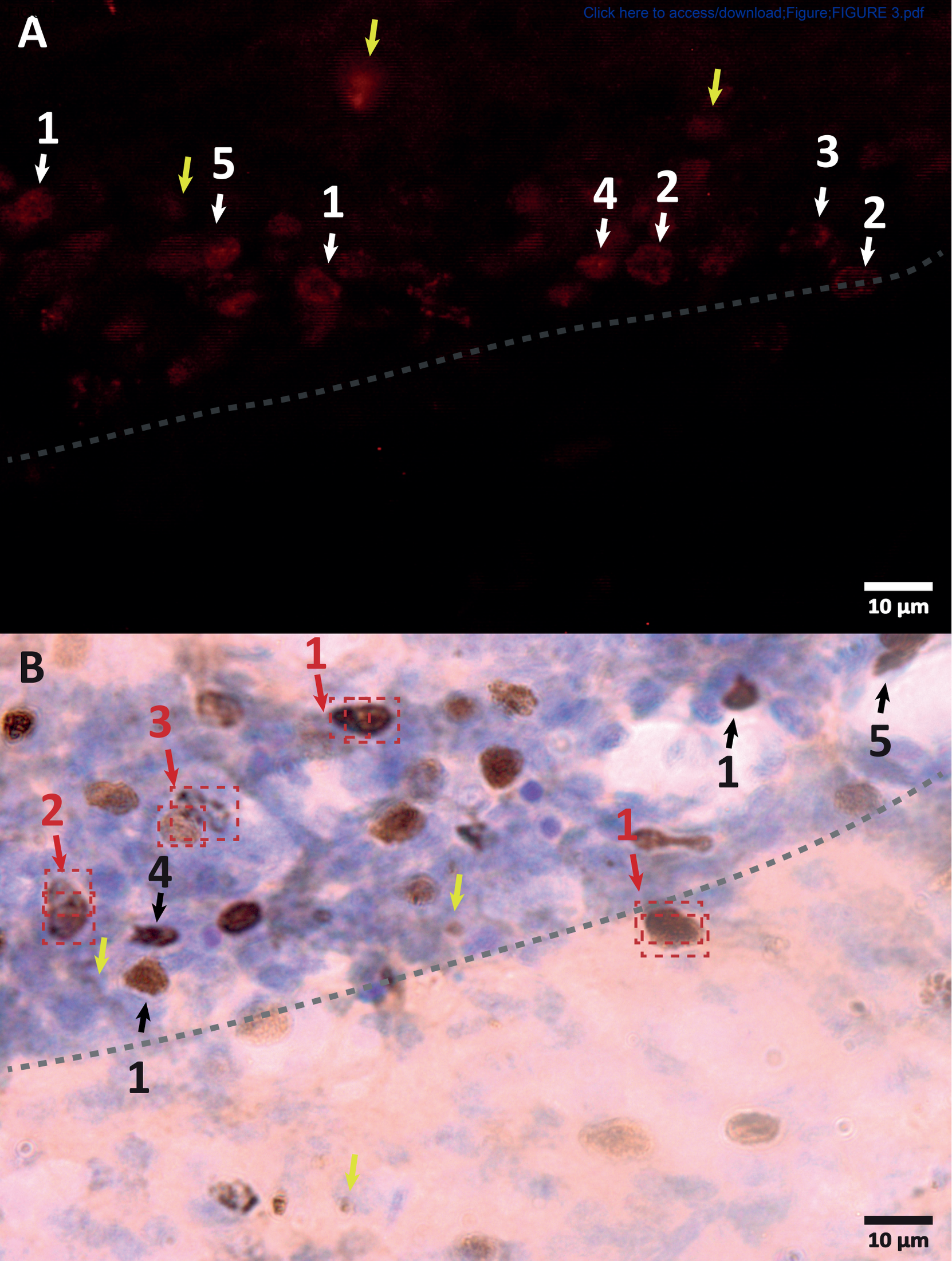


FIGURE 4

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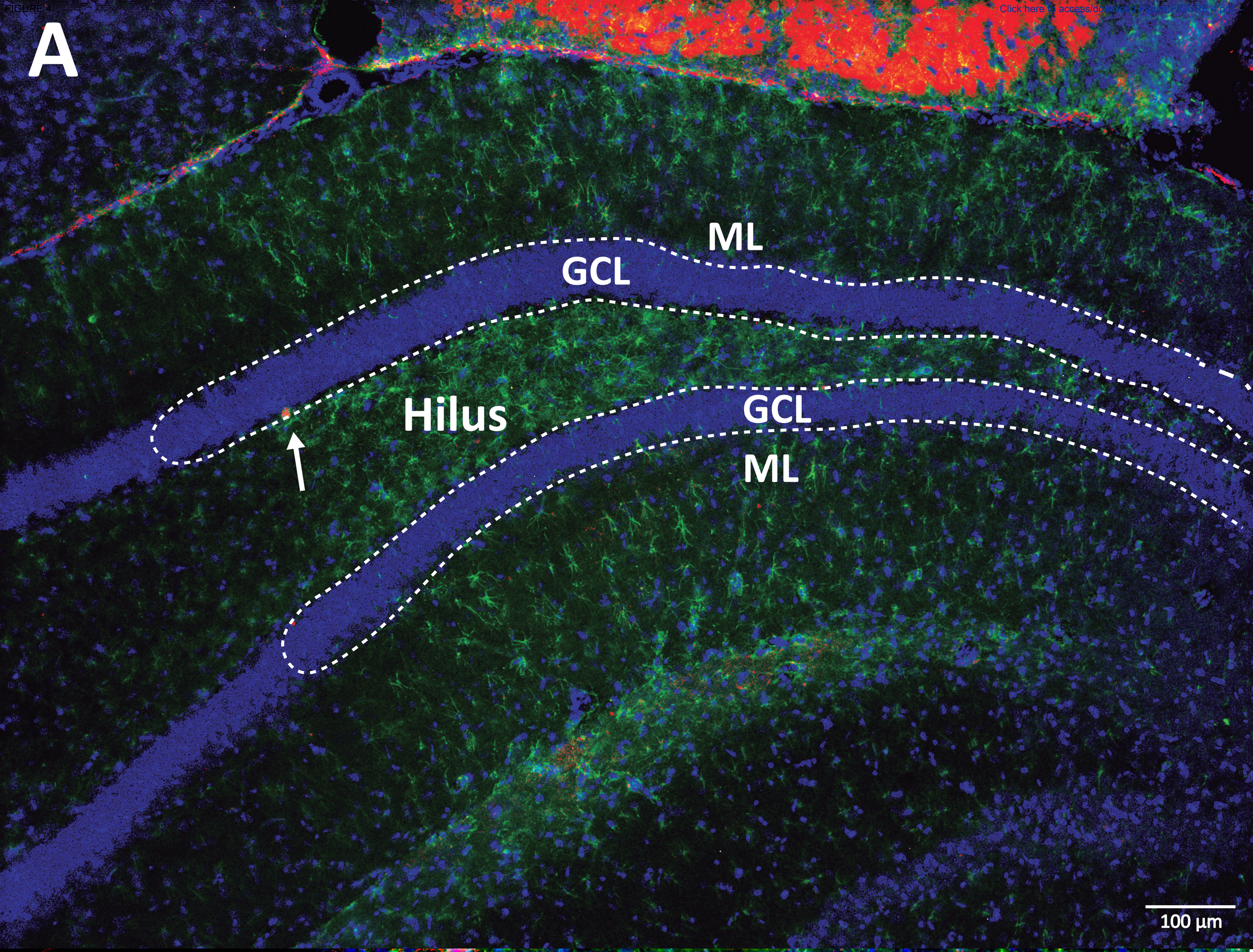
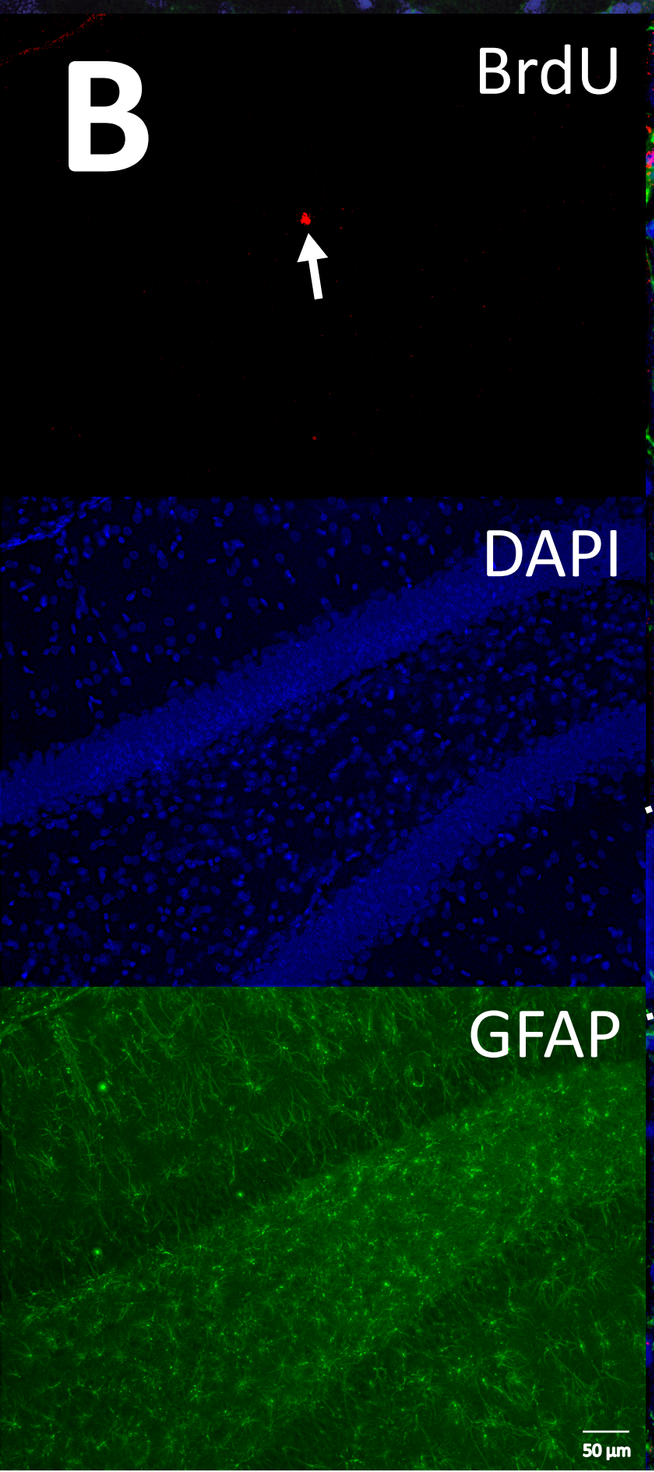




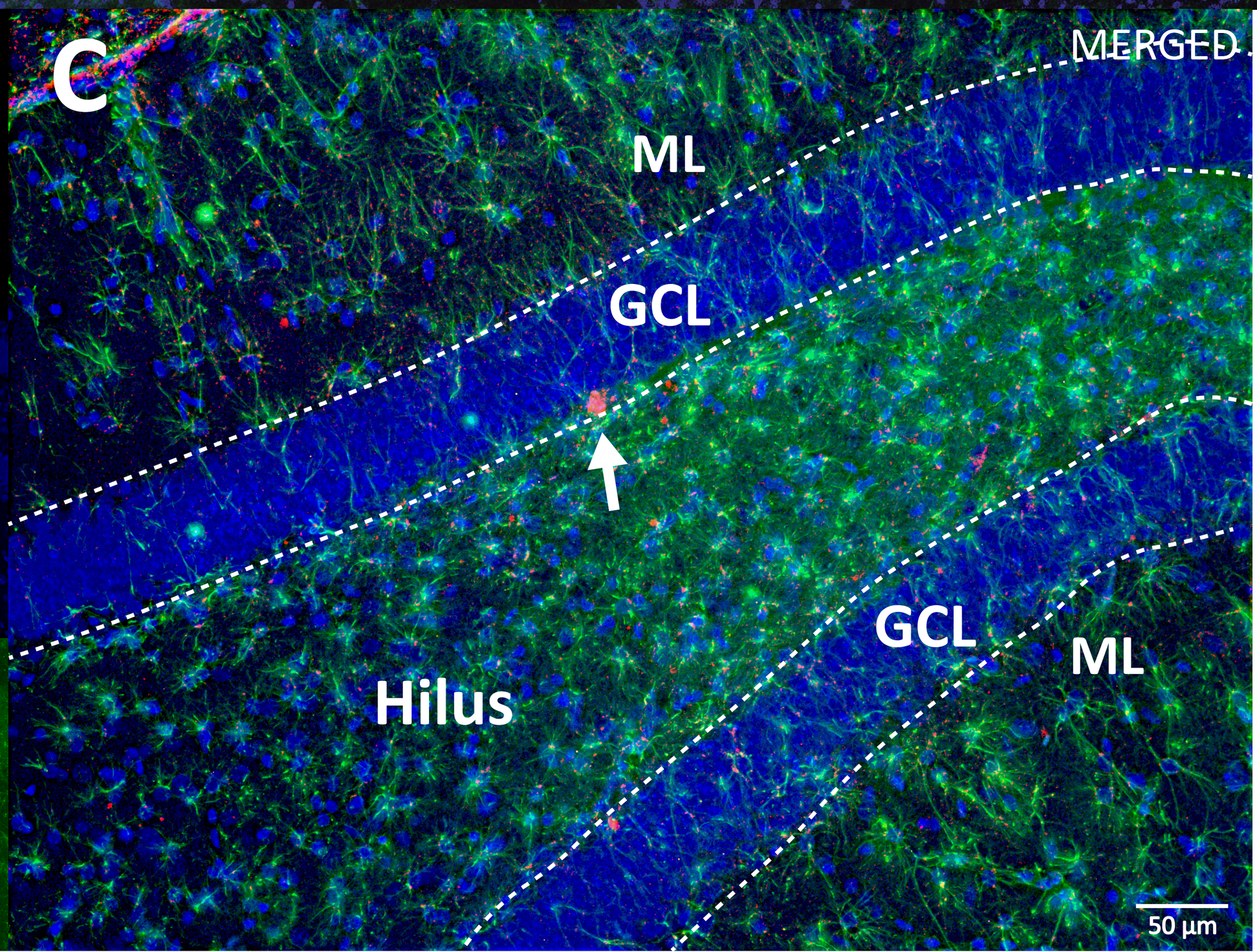
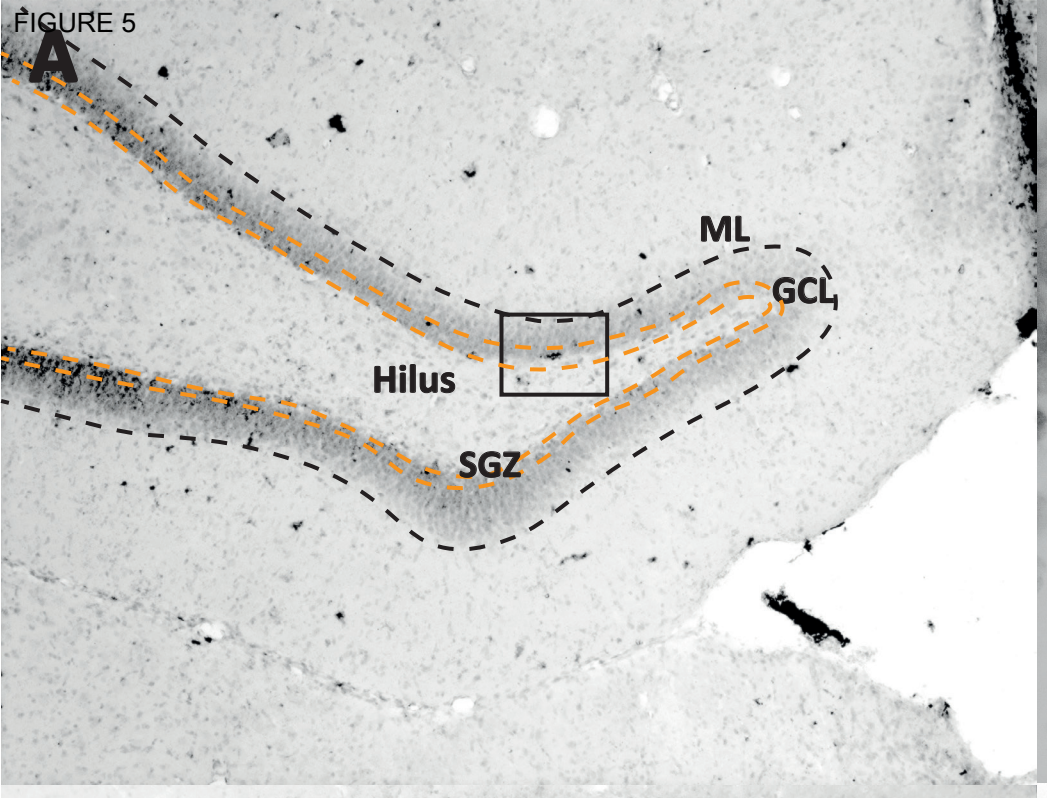
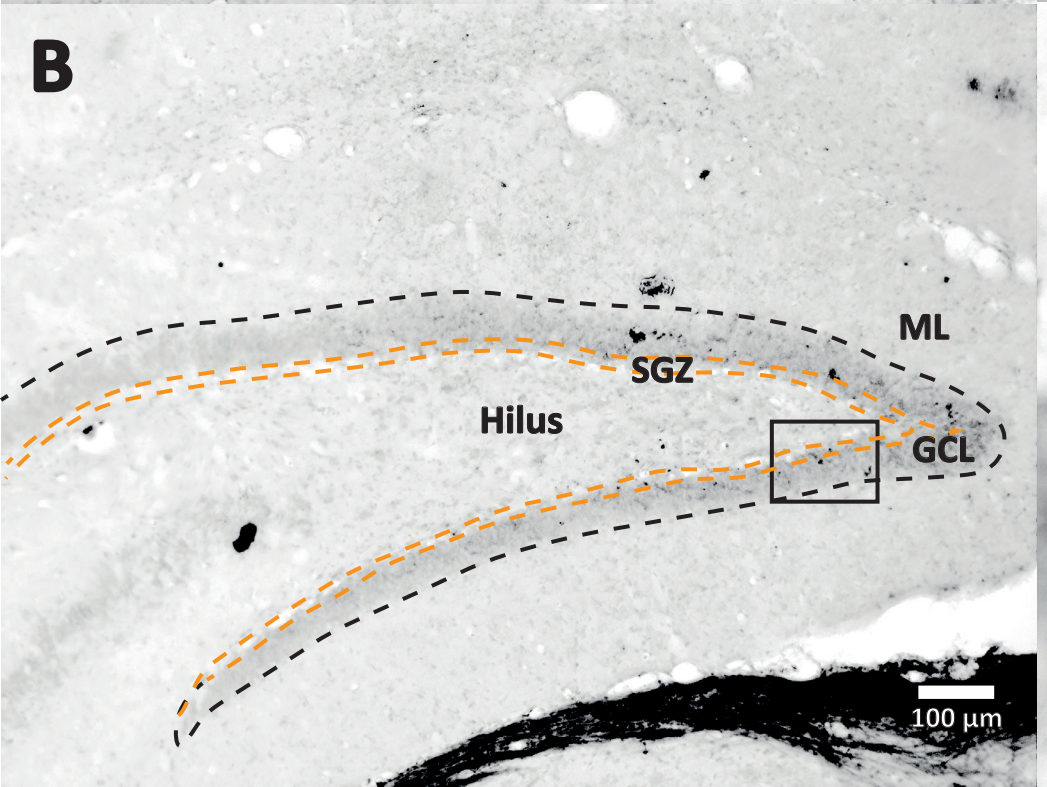
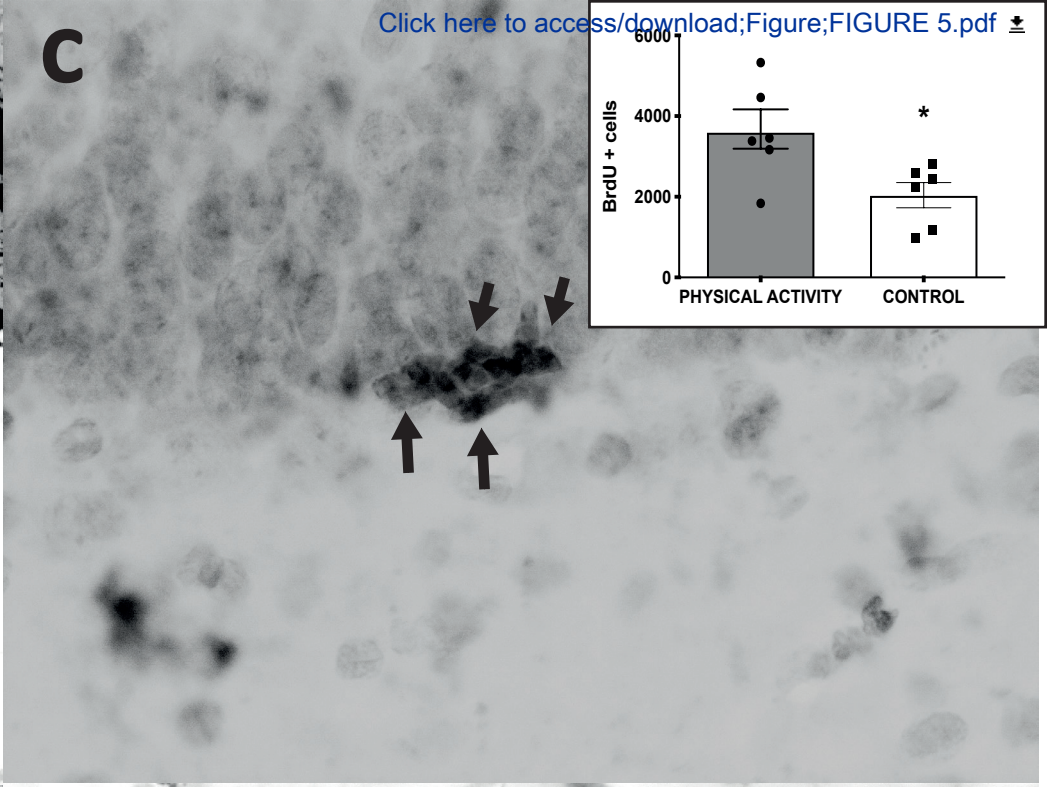
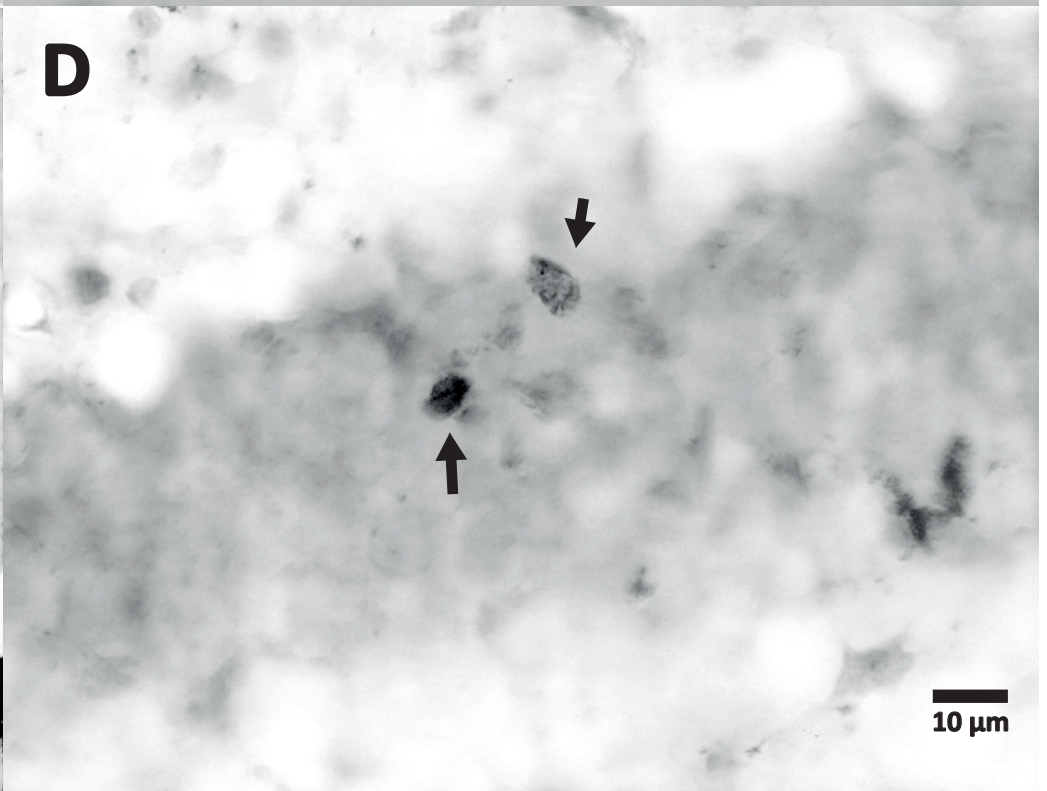


FIGURE 5









VIDEO 1

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VIDEO 2

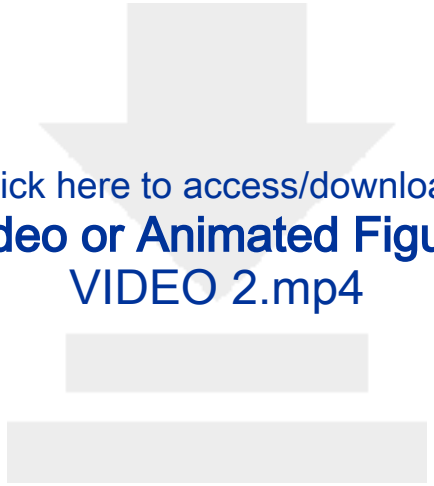

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TABLE 1

Method	Specific Method	Advantages
Indirect Detection Method	Peroxidase reaction with DAB	1. Higher sensitivity than the direct detection and indirect fluorescence method. 2. Higher resistance to Photobleaching than fluorochromes. 3. Lower cost than fluorescence detection method
	Fluorescence	1. Best and easiest for Multiplexing with more color dyes. 2. Best for Co-expressed targets in the same cellular space. 3. Better Dynamic Range for simultaneous scarce and high abundant targets on the same tissue. 4. No Additional Steps.
Signal Amplification Method	Avidin-Biotin Complex (ABC)	1. Higher sensitivity than the direct and indirect detection method. 2. Reduce background
	Labeled Streptavidin-Biotin (LSAB)	1. Higher sensitivity than the direct and indirect detection method. 2. More substantial tissue penetration than the ABC method. 3. Reduce background
	Not additional amplification method	1. Lower cost. 2. No additional steps. 3. Ideal for high abundant targets.

Disadvantages
1. Difficult for Multiplexing with fewer color dyes. 2. Complicated for Co-expressed targets in the same cellular space. 3. Reduced Dynamic Range for simultaneous scarce and high abundant targets on the same tissue.
1. Lower sensitivity than the indirect peroxidase reaction with DAB method. 2. Weak resistance to Photobleaching over time. 3. More expensive.
1. Additional Steps. 2. More expensive than not amplification.
1. Additional Steps. 2. More expensive than the ABC method.
1. Lower sensitivity: problematic with no abundant targets.

TABLE 2

Microscope Type:	Epifluorescence Microscope Olympus BX53			
Light Source:	High-pressure 130 W mercury arc lamp (U-HGLGPS)			
Acquisition Software:	CellSens Standard			
Filters Sets:	**Catalog Number**		**Excitation Range**	**Dichromatic mirror**
	U-FUW		340 - 490 nm	410 nm
	U-FBW		460 - 495 nm	505 nm
	U-FGW		530 - 550 nm	570 nm
Camera:	**Model:**		CCD-camera UC50	
	Spectral range:		290 – 1000 nm	
	CCD chip size:		2/3 in, 2588 (Width *7) X 1960 (He	
	Pixel Size:		3.4 X 3.4 µm	
Fluorochrome:	**Name**		**Excitation Wavelength (nm)**	**Emission *3 Wavelength (nm)**
	4, 6-diamidino-2-phenyl-indole HCl (DAPI)		345	455
	Tetramethylrhodamine-isothyocyanate (TRITC)		541	572
	Fluorescein-isothiocyanate (FITC)		494	519
	Cy3		552	565
Mounting Medium and immersion oil:	**Name**			**Indexof refractio**
	Air (nothing between the slide and the lens)			1.00
	Antifade Mounting Medium with DAPI			1.4
	Permount Mounting Medium			1.5
	Low autofluorescence immersion oil (MOIL-30 Type F)			1.5
Magnification Lens (Plan Fluorite)	**Magnification**	**Numerical Aperture (NA) *2**	**Resolution (µm)**	**Image pixel spacing (nm) *5**
	4X	0.13	2.12	850
	10X	0.3	0.92	340
	20X	0.5	0.55	170
	40X	0.75	0.37	85
	100X	1.3	0.21	34

Suppression Range
420 nm
510 nm
575 nm

eight ***8***) pixels

Emission Color
Blue
Red
Green
Red

n of the media *1

Slice spacing Z-axis (nm) *6
3000
3000
2000
1000
1000

TABLE OF MATERIALS

Click here to access/download;Table of Materials;Table of Materials.xlsx

Name of Material/ Equipment	Company
REAGENT PREPARATION AND SETUP	
Donor Horse Serum	BioWest
Paraformaldehyde reagent grade, crystalline (PFA)	Sigma-Aldrich
Potassium Chloride	Sigma-Aldrich
Potassium Phosphate, monobasic	J.T Bker
Sacarose	J.T Baker
Sodium Chloride	Meyer
Sodium Hydroxide	Sigma-Aldrich
Sodium Phophate Dibasic	Sigma-Aldrich
Triton-x 100	Sigma-Aldrich
THYMIDINE ANALOGUE BRDU ADMINISTRATION	
5-Bromo-2'-deoxyuridine, BrdU	Sigma-Aldrich
23–27G hypodermic needle	BD PrecisionGlide
Saline solution	PiSA
Syringes 1 mL	NIPRO
TISSUE PREPARATION	
15-ml polypropylene conical tube	Thermo Scientific
50-ml polypropylene conical tube	Thermo Scientific
Dissecting tools	
Guillotine	Stoelting
Microtome Cryostat	MICROM
Netwell 15 mm polyester mesh membrane inserts	Corning
Netwell plastic 12-well carrier kit	Corning
Netwell plastic 6-well carrier kit	Corning
Perfusion pump	Cole-Parmer
Shaker	IKA
IMMUNOSTAINING	
Cresyl violet	Sigma-Aldrich
DAB Peroxidase (HRP) Substrate Kit (with Nickel), 3,3'-diaminobenzidine	Vector Laboratories
Hydochloric Acid	J.T.Baker
Hydrogen Peroxide, 50%	Meyer
Permount Mounting Medium	Fisher Chemical
VECTASHIELD Antifade Mounting Medium with DAPI	Vector Laboratories
VECTASTAIN® Elite® ABC Kit Peroxidase (HRP)	Vector Laboratories
Primary antibodies	
Anti-GFAP antibody produced in rabbit	Sigma-Aldrich
Monoclonal Anti-BrdU antibody produced in Mouse	Sigma-Aldrich
Secondary antibodies	
Biotin-SP (long spacer) AffiniPure Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoResearch

Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, HRP	Invitrogen
Goat Anti-Mouse IgG (whole molecule), TRITC	Sigma-Aldrich
Goat Anti-Rabbit IgG (H+L) Cross-Adsorbed, FITC	Invitrogen
Streptavidin, Cy3	Vector Laboratories
IMAGING AND ANALYSIS	
Computer	Dell Computer Company
CCD-camera	Olympus
CellSens Standard software	Olympus
Epifluorescent microscope	Olympus
High-pressure 130 W mercury arc lamp	Olympus
low autofluorescence immersion oil	Olympus
Micro cover-glasses (VWR, cat no 48404 454; 24 60 mm)	
Microscope slides (VWR, cat no 48323-185; 76 26 mm)	
U-FBW filter cube	Olympus
U-FGW filter cube	Olympus
U-FUW filter cube	Olympus

Catalog Number	Comments/Description
S0900	Blocking and incubation solutions in PBS
P6148	toxic, flammable
746436-500G	
3246-01	
4072-01	
2365-500G	
S5881-500G	Corrisive, to calibrate pH
S9763-5KG	
T8787	
B9285	toxic (mutagenic, teratogenic)
30130032	
339650	
339652	
HM525	
3477	pre-loaded in 12-well culture plates
3520	for 15 mm polyester mesh membrane inserts
3521	
7553-70	
ROKCER 3D Digital	
C5042	1%, Light sensitive
SK-4100	carcinogenic, light sensitive
9535-05	Corrosive, to calibrate pH
5375-1L	Toxic, oxidative
SP15-500	
H-1200	Light sensitive
PK-6100	enzymatic, avidin/biotin based amplification system
HPA056030	1:500
B2531	1:250
715-065-151	1:250

G-21040	1:1000
T5393	1:250
F-2765	1:250
SA-1300	1:250
T8P8T-7G8MR-4YPQV-96C2F-7THHB	For controlling and monitoring protocols' processes
UC50	
cellSens Standard Edition	Acquisition Software
BX53	
U-HGLGPS	
MOIL-30 Type F	
U-FBW	excitation 460 - 495 nm, dichroic mirror 505 nm, suppression 510 nm
U-FGW	excitation 530 - 550 nm, dichroic mirror 570 nm, suppression 575 nm
U-FUW	excitation 340 - 490 nm, dichroic mirror 410 nm, suppression 420 nm

Vineeta Bajaj, Ph.D.
Senior Review Editor
Journal of Visualized Experiments

16 July 2020

Dear Dr. Vineeta Bajaj,

Subject: Immunohistochemistry Techniques to Analyze Cellular Proliferation and Neurogenesis Using the Thymidine Analog BrdU. Manuscript No. JoVE61483R1.

Thank you for your email, enclosing the editors and reviewers' comments. We have carefully reviewed the comments and have revised the manuscript accordingly. Our responses are given in a point-by-point manner below. The changes to the manuscript have been tracked to identify all of the manuscript edits. The corrections and suggestions provided by you and the reviewers helped improve the paper. We hope the revised version is now suitable for publication, and we look forward to hearing from you in due course.

Sincerely,
Mario Buenrostro-Jauregui, Ph. D.
Universidad Iberoamericana
México City
México
mario.buenrostro@ibero.mx

General Statements about the Revised Manuscript

We strived to cover most of the reviewers' comments. We addressed those comments that we felt were the most relevant and those who stuck to our article's focus. In other cases, we justify the reasons for noncompliance in the Point-by-Point Response to Comments section of this letter (see below). We also performed minor modifications throughout the article adds to those kindly recommended by reviewers to improve clarity and accuracy. Changes in the manuscript were agreed on by all authors. An academic English editor service was hired to revise and proofread the manuscript. All the changes in the manuscript by both the editor and us were tracked. You will find below a point-by-point response to yours' comments. Regular font style is used for featured comments, and italics have been used to show our responses.

Point-by-Point Response to Editorial Comments

Editorial Comments

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached version for revision.

2. Please address all the specific comments marked in the manuscript.
3. Once done please ensure that the highlight is no more than 2.75 pages including headings and spacings.

The manuscript needs a thorough proofreading.
Response: This issue was addressed. The manuscript has been edited by an academic English Editor service.

Please ensure the Introduction to include all of the following:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application
Response: We reworded some parts of the Introduction section.

How where? “The patients received the injection”
Response: We added the info as follow “The patients received the BrdU injection (250 mg intravenous).” (see page 2, line 82).

Please remove the redundancy and make the protocol crisp.
Response: This issue was addressed.

Please use complete sentences throughout.
Response: This issue was addressed.

Please do not use personal pronouns in the protocol section
Response: This issue was addressed.

Please use S.I. abbreviations throughout so ml will be mL, gram is g, Liter is L, hour is h, minute is min, etc. Please do not include a period after the abbreviation.
Response: This issue was addressed.

Once all changes have been performed, please ensure that the highlight is no more than 2.75 pages including headings and spacings.
Response: This issue was addressed.

If small volume is preferred why is the dilution performed? The sentence above state 0.5 mL/ rat , here it ‘s 20mg/mL. Please check this discrepancy.
Response: There is no discrepancy; they are two different things. Step 1.1.1 states, "Make enough volume calculating at least 0.5 mL/rat per injection," which refers to the volume that will be injected to each rat. While in the Note states, "dilute the dose of 50 mg/per kg of

body size in a final solution of 20 mg of BrdU in 1 mL." The above refers to the final concentration of the solution, not the volume to be injected. We understand that repeating the instruction can generate confusion, so we decided to withdraw the second one. The final Note is "It is preferable to inject a small volume to minimize the suffering of the animals." (see page 6, line 250).

Please change this to Double distilled throughout the manuscript.
Response: This issue was addressed.

What is this used for? Where is C? Please expand the term "1.10. ABC solution"
Response: We reworded the sentence as follows "1.10. Avidin-Biotin Complex (ABC) solution." (see page 8, line 323).

Which part of the body? "2.2. Administer intraperitoneally (i.p.)"
Response: Intraperitoneal injection is the injection of a substance into the animals' peritoneum. It is a very common administration way. We reworded the sentence as follows "2.2. Administer the BrdU solution (50 mg/kg) intraperitoneally (i.p.) using a proper gauge needle (e.g., 23 G) and syringe (e.g., 1 mL) for the animal weight (e.g., 350 g)." (see page 9, line 354).

Citation? "Different routes could be used to administrate the BrdU solution."
*Response: We added a citation "*Taupin, P. BrdU immunohistochemistry for studying adult neurogenesis: Paradigms, pitfalls, limitations, and validation. *Brain Research Reviews*. **53** (1), 198–214, doi: 10.1016/j.brainresrev.2006.08.002 (2007)."

Please do not make too many sub headings.
Response: We have eight subheadings that indicate very different stages of the protocol; removing them can generate more confusion between the steps. We removed the subtitles that could be integrated into the body of the text.

Please reword for clarity. What kind of physical activity was performed? When was the time point of BrdU injection? "NOTE: Three-month old rats underwent a voluntary physical activity protocol for seven days. Rats were injected three times with BrdU, as described above in step 2, with 12 h between each other. To complete three cell cycle divisions, 8 h after the last BrdU injection continues to step 3."
Response: We reworded the paragraph as follows "NOTE: Three-month-old rats underwent a voluntary physical activity protocol (endless wheel) for seven days. On day 6, rats were injected with BrdU (step 2), and every 12 h until complete three injections. Continues to step 3 after eight hours of the last BrdU injection." (see page 9, line 365).

Blood is drained out and the solution becomes clear. "Using a pump or gravity, perfuse (flow rate 5-7 mL/min.) 0.1 M PBS until the draining blood becomes clear."

Response: We reworded the sentence as follows "Using a pump or gravity, perform cold perfusion (flow rate 5-7 mL/min) with PFA solution to fix the tissue until the tail becomes rigid." (see page 9, line 385).

Added here please check. "to fix the heart until the tail becomes rigid."
Response: We reworded the sentence as follows "to fix the tissue until the tail becomes rigid." (see page 9, line 385).

Which solution PBS or PFA? "NOTE: Usually, a 300 g rat requires around 100-150 mL of the solution."
Response: We reworded the sentence as follows "NOTE: Usually, a 300 g rat requires around 100-150 mL of the PFA solution." (see page 9, line 392).

Decapitate? "3.4.1. Remove the animal's head"
Response: We reworded the sentence as follows "3.4.1. Decapitate, and gently extract" (see page 10, line 398).

Do you visually look for something when checking the degree of fixation. "NOTE: Do not over-fixate, because tissue could lack staining due to the unavailability of antigens."
Response: We reworded the sentence as follows "Do not over-fixate (more than 48 h), because tissue could lack staining due to the unavailability of antigens." (see page 10, line 402).

What is being done here? "3.4.2. Immerse the brain into a conical tube with a 30% sucrose in 0.1 M PBS solution (35 mL) for approximately 1-2 days at 4 °C until the brain sinks in the tube."
Response: We reworded the paragraph as follows "3.4.2. Prepare a 100 mL of 30% sucrose solution, adding 30 g of sucrose to 70 mL 0.1 M PBS solution under constant stirring. Add 0.1 M PBS solution until 100 mL. Immerse the brain into a conical tube with a 30% sucrose solution (35 mL) for approximately 1-2 days at 4 °C until the brain sinks in the tube." (see page 10, line 405).

This note is redundant and can be removed. Citing ref 13 is ok. "NOTE: There is a diversity of instruments as a viable option to cut the thin (40) brain slices. The rocking microtome, sliding microtome, vibratome (which does not require freezing the tissue), and the cryostat-microtome (which requires freezing the tissue) are the two commonly used techniques. In this case the second type was used because of its availability in the laboratory. Using a cryostat-microtome requires training and guidance. Carefully review the manual guidelines of the instrument. To cut brain section, read further detailed instructions, see ref.14."
Response: We reworded the note as follows "NOTE: Using a cryostat-microtome requires guidance and training. For detailed instructions, see Revilla & Jones (2002)." (see page 10, line 41).

Is this whole brain from step 3.4.2? Is there a washing step in between? “3.5.1. Submerge the brain into -80 °C iso-pentane and keep it at -80 °C for 10 min. Place the brain in an embedding matrix on a cryostat-microtome plate.”
Response: We reworded the sentence as follows “3.5.1. Submerge the whole brain (from step 3.4.2) into -80 °C iso-pentane and keep it at -80 °C for 10 min. Place the brain in an embedding matrix on a cryostat-microtome plate”. Not washing step is needed. (see page 10, line 421).

Please include the step numbers. Also notes cannot be filmed so please remove the highlight throughout.
Response: This issue was addressed.

Reworded for clarity please check. “NOTE: Perform step 4.3.1 to 4.3.4 on the day 1.”
Response: This issue was addressed. We are ok with change.

Removed the note and added here. “4.3.5. On the day 2, Rinse 3 times, 10 min each, into 0.1 M PBS.”
Response: This issue was addressed. We are ok with change.

How do you visually identify? Will you observe this in all brain sections? “identify the dentate gyrus properly”
Response: Since we don't have a lot of space to describe in detail instructions to identify the DG. We reworded the sentence as follows “8.1. To quantify cells, first, identify the dentate gyrus properly with the 4X magnification lens (for further instructions on DG anatomical detail, see Amaral et al., 2002)”, and added the ref 15. (see page 16, line 665).

Please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Response: We reworded some parts of the Discussion section.

Reviewer #1:
Manuscript Summary:
The authors satisfactorily answered all my concerns. They have improved the quality of their images, which was the main flaw of the previous version of the manuscript.

Reviewer #4:
Manuscript Summary:

This submission detailed the entire process of BrdU detection of new neurons, from BrdU injection to microscopy and quantification. Overall, this is an ambitious task to take on and the authors do a pretty good job of creating a rigorous protocol. The title suggests that this submission will only cover the IHC detection for BrdU. The authors may want to adjust the title to include the other methodologies. That said, the IHC section contained the most clear and comprehensive description of methodology. BrdU injections, perfusions, and thin tissue sectioning were covered in comparably less detail, at levels closer to what you can already find in publications. The microscopy section probably requires the most attention, as it was a bit technical while also lacking in detail. In fact, an entirely separate submission could be devoted to microscopy alone, hence the current protocol being a bit ambitious. Below are some additional considerations.

Major Concerns:

1) The introduction provided a great description of procedural history and comparison with more modern techniques. However, it could be useful for the authors to also add some details about how long BrdU will circulate the blood before metabolization. This could be useful information to individuals accessing this document for experimental design purposes.

Response: We added the following sentence to introduction section: "It is essential to know that BrdU is present in a saturated concentration in mice and rat serum for 15 and 60 min, respectively, after intraperitoneal administration, then drops rapidly until undetectable levels at 60 and 120 min respectively." (see page 3, line 128).

2) 1.12 The authors may want to add a disclaimer to their cresyl violet solution. Their current methodology may come out a bit dark for a counterstain with BrdU, thereby obscuring the ability to accurately count cells. An experimenter may want to play with nissl concentrations before committing to a counter stain.

Response: We added a note as follows "The user is encouraged to test different concentrations of the cresyl violet solution before using it on valuable tissue samples. The result may be dark for counterstaining with some tissue samples, which may decrease the ability to count BrdU positive cells accurately." *(see page 8, line 340).*

3) In 3.5.1 do the authors mean -80 or -20C? Our experience with rapid freezing brains to -80 is that they crack and cause damage to the tissue. Furthermore, if they are being placed at -20C for sectioning, it's not clear why they should be at -80F, given the risk of fast freezing tissue damage.

Response: We have never experienced tissue fracture when we immerse the tissue at -80 ° C in iso-pentane bath. In any case, we added a note as follows "Under certain conditions, a fast brain freezeat -80 °C can cause fracture or damage to the tissue. The user should be aware of this problem. If so, adjust a temperature of -20 ° C in iso-pentane bath to freeze the brain." (see page 10, line 424).

4) 3.5.2 Protocol says store in cryoprotectant for up to one month. We've stored tissue for years and had excellent results. Perhaps mention that it's possible to store for longer, so new experimenters accessing this protocol don't discard tissue older than a month.
Response: We added a note as follows "It is possible to store brain sections for years under the right conditions." *(see page 10, line 432).*

5) I was unable to view the figures, as my PDF would crash every time it would try to load the pictures. I've never experienced this before on a PDF. I have well equipped computer, and tried restarting my program and computer, so I'm not sure what's going on.
Response: It is something strange to happen. In any case, we have rebuilt the image files to solve a possible coding error in the image file construction.

6) Please note various minor editing errors, like 8.2.2
Response: This issue was addressed. The manuscript has been edited by an academic English Editor service.

Reviewer #5:
Manuscript Summary:
Journal of Visualized Experiments
Immunohistochemistry techniques to analyze cellular proliferation and neurogenesis using the thymidine analog BrdU
Buenrostro-Jauregui M. et al

The concept of neurogenesis in the adult mammalian brain has met with skepticism first; but in the end, has also resulted in important new technical and scientific insights; i.e. showing that DNA synthesis and incorporation of thymidine analogue BrdU is not confined to de novo neurogenesis, but may also occur in damaged and dying neurons.
The manuscript by Buenrostro-Jauregui et al. describes extensively the staining method, and procedures, namely of BrdU, and its relevance in defining adult neurogenesis in the dentate gyrus. I wasn't aware of its difficulties that much; but it is overall a good idea to bring these important method to a broader audience. I am aware that this already is a revised manuscript, and I hope my suggestions won't collide with previous reviewers. Please see all comments in detail below.

Comments:
For the abstract (p2 li46 when DNA is mentioned) and the introduction (p2 li102), it seems very important to mention the acid denaturation (HCL) step here (which opens the nucleus; since this is, to my knowledge, the main reason why people who are familiar with IHC in general may not be able to detect BrdU at first).
Response: We added a mention in the abstract as follows "For instance, there is a necessary a step to reach the DNA, which consists of denaturize it with HCL." (see page 2, line 51); and in the introduction as follows "On the other hand, BrdU can be found in the cellular nucleus since it is incorporated into the DNA during S-phase by competition. Therefore, a crucial step is the DNA denaturalization with HCL, which opens the DNA bonds to allow the BrdU antibody access to the BrdU within the DNA." (see page 3, line 125).

Furthermore (s. Discussion, p17 li725-30), although BrdU labels mitotic cells of a mainly neuronal lineage in the SVZ, SGZ, it is not per se a neuronal marker; to detect those one has to double/triple stain for markers of the neur(on)al lineage (e.g. Kempermann 2004; please revise the sentences). In addition, BrdU can be found throughout the entire brain, as it not only marks neurogenic niches but also the progenitor cell in non-neurogenic regions (NG2+ cells, mainly found in pairs).
Response: We added some sentences to solve this issue as follows "As mentioned before, BrdU staining is the most commonly used method for adult neurogenesis research to detect cell proliferation. However, the use of BrdU as a marker has limitations and pitfalls. The first one is that BrdU is a cell cycle marker. Therefore, double or triple staining must be performed to identify the cell fate, and include cell markers to detect the specific developmental stage of the cells labeled." (see page 20, line 847).

For the BrdU antibody used, I am wondering why you use mouse; the main and widespread one is rat monoclonal BrdU from (now) Abcam; it is important that it contains BU1/75 (ICR)1. (Rat is also a good choice in terms of co-labeling since additional antibodies mainly derive from i.e., mouse or rabbit.)
Response: As we used rats for our experiments, and under the general premise that the primary antibody is increased, the animal should be different from the species used in samples to avoid cross-reactivity, therefore, we used anti-mouse antibody. However, since the brain tissue was perfused, you might expect to see low non-specific labeling by the anti-rat secondary antibody in the brain tissue and blood vessels. Therefore, we will test this particular antibody (Anti-BrdU antibody [BU1/75 (ICR1)] ab6326), thanks for your valuable suggestion. We used the monoclonal anti-BrdU (mouse IgG1 isotype) antibody (B2531 SIGMA) which is derived from the BU-33 clone. This antibody has widely been used to investigate cell proliferation specifically in hippocampal cells. Nevertheless, we appreciate the valuable suggestion.

1. Reagent preparation and setup
1.1.2.1 /2 e.g., a pre-heat or water bath is not needed, a strong vortex is enough; however, in use of larger amounts you will probably have to heat it up
Response: We added a note as follows "NOTE: Pre-heat the saline solution at 45-50 °C in a water bath for volumes bigger than 1ml." (see page 6, line 255).

IMPORTANTLY, when BrdU is dissolved in saline, it has to be filtered for sterile injections, and kept in the dark; as of the first manuscript version, you had written that an 'in saline diluted BrdU' can be used for 24 hours. This is correct, and I would mention it!
Response: We added a note as follows "It is recommended to prepare the BrdU solution immediately before use. However, the solution is stable for 24 h under rt. Filter the solution with a syringe filter for sterile injection. Please protect it from light." (see page 7, line 266).

For PBS, and 'horse' serum, respectively, I would mention that you can alternatively use 1 x TBS, or any other serum (bovine or donkey), respectively
Response: We added a note as follows "NOTE: Alternatively, TBS can be used instead of PBS. Any other serum different from the host's antibodies and experimental tissue is suitable." (see page 7, line 281).

For PBS+, ++, e.g., 3% serum and a final conc of 0.1% Triton are enough (4.1.4, 5 30 min blockage in PBS+, and antibodies in PBS+)
Response: We are sure that alternative concentrations for Triton work fine. However, this protocol includes times and concentrations for all working solutions tested and used in experiments that are carried on in our laboratory. As mentioned in the discussion section, it is recommended that every research group try to make their tests with their tissue, antibodies, and particular conditions. We recommend that every researcher group needs to develop a standard procedure to test every new antibody and tissue used (see page 20, line 871).

1.5 CPS does not need to be filtered, and slices are good for years at 4 deg
Response: In order to have sterile solutions is preferable filter the CPS. Regarding the time that CPS preserve the slices, we changed the paragraph as follow "1.5. Cryoprotectant Solution: Prepare 1 L, add 250 mL of ethylene glycol, 250 mL of glycerol, stir constantly until mixed. Slowly bring to 1 L with PBS. Filter with grade 4 (20-25 µm) filter paper. Store at 4 °C or RT for up to 1 year." (see page 7, line 287).

1.6 PFA 4%, is now ready-to-use commercially available (Formaldehyde solution 4%, buffered; Merck, Sigma), I would mention this (although, of course, it may not be available everywhere)
Response: We added a note as follows "PFA ready-to-use solution is commercially available." (see page 7, line 291).

Secondly, there is no need to perfuse with PFA anymore (just Saline or PBS), the 'overnight/24 hrs fixation step is enough - this is also good to minimize animal usage/experiments, since one brain can be used for several methods; e.g., one hemisphere for protein (IHC), and the other for RNA analysis (or other methods, e.g., PCR or western blot or FACS)
Response: We added a note as follows "The tissue fixation (3.3.2 step) could be omitted. Thereby, the brain can be used on several methods and minimize animal usage for experiments." (see page 9, line 392).

1.8 (4.2.3) 2N HCL, the calculation is wrong (3 in 47)?! It should be, in 50 ml, 8.25 HCL into 41.75 ml H2O; 20 min at 37 deg is enough
Response: We corrected this issue as follows "Prepare 50 mL by slowly adding 8.25 mL of 12 N HCL (concentrated stock solution) to 41.75 mL Double distilled H2O under constant stirring." (see page 7, line 306).

1.9 (4.1.2) for the peroxidase step, H2O2 is light sensitive, this staining step should be in the dark (cover the well plate with a foil or box); the common dilution is 0.6% (40 ml TBS/PBS and 0.8 ml 30% H2O2)
Response: We corrected this issue and added a note as follows "Please keep it in the dark due to H2O2 is light sensitive. PBS or TBS can be employed instead of water." (see page 8, line 319).

1.10 for ABC, (and also 1.11 DAB), I would in addition state the 'real' amount, not just 'drops'; the common dilution for ABC is 9 µl A and 9 µl B into 1 ml (TBS or PBS); for DAB, 500 µl in 40 ml, 12 µl H2O2, 200 µl NiCl2 (8%)
Response: We added this information following the manufacturer's user guide (see page 8, line 323, and page 8, line 329).

3. 'Note' on voluntary wheel running, i) why mentioning a three-times injection regime here, it is not relevant for the review (the protocol); however, ii) three-times e.g., is every 6 to 8 hours (Klempin et al., 2013), that makes it a total of 24 hrs corresponding to catch all proliferating cells possible in mice iii) rats do not love running as much as mice
Response: The additional information on the injection was a request from the journal editor. As the reviewer mentions, rats run less than mice. However, in our case, the voluntary physical activity protocol helps the rats to run for longer periods. However, some rats show little activity. We jsut used a 50% of animals that run longer.

3.5 please check (40) "µm", which is missing a few times here
Response: This issue was addressed.

4.1.3/4 IMPORTANTLY, following the HCL step, slices should be de-acidified; therefore slices should be rinsed for 10 min in 0.1 M Borate Buffer (8.5 pH)
Response: We added this step to all HCL steps (see page 11, line 454; page 12, line 491; page 13, line 531; and page 13, line 565).

4.1.8 for washing/rinsing following DAB staining, normal tap water can be used; this is the most important step, e.g., to reduce the background, thus 3x rinse in water should be at least 15 min each!
Response: We added this step to all washing/rinsing following DAB staining steps (see page 11, line 470, and page 12, line 510).

Overall, the ABC-DAB step is very important and commonly used (4.2 as the mainly used protocol); streptavidin is not necessary anymore, the commercially available biotin antibodies are very good; thus, I would suggest to summarize the few staining variants for day 2 at the end as 4.1.6.1 and 2 ...) instead of writing all steps?!
Response: We appreciate the reviewer's suggestion, however to avoid misunderstandings or confusions to the reader, we decided to leave the protocols completely for a better step-by-step follow-up procedure.

Mounting medium is best self-made using PVA(Polyvinyl alcohol)-DABCO
Response: We added a NOTE as follow "Self-made mounting medium using PVA(Polyvinyl alcohol)-DABCO can be employed." (see page 15, line 648).

For double, triple labeling, all primary antibodies can be added at the time for overnight staining at 4 deg
Response: We present the alternative suggested by the reviewer in step 6.1. Simultaneous multiple immunostainings. (see page 14, line 609).

Minor comments:

all staining steps need to be on shaker; and it's a 'free floating' protocol
Response: This issue was addressed.

please check 'thymidine analog' which you often wrote with 's'
Response: This issue was addressed.

p2 li86, Nestin is a stem/progenitor marker (which together is called 'precursor'), and "the nestin-GFP mice allows to determine 'early' developmental steps' in ..." fate decision towards neuron.
Response: This issue was addressed.