

BrdU assay for neurogenesis in rodents

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Neurogenesis within the adult central nervous system is demonstrated using an exogenous cell tracer, 5'-bromo-2'-deoxyuridine (BrdU), in combination with endogenous neuronal markers. Specific primary antibodies raised against these markers are widely available and their visualization is possible with the use of fluorescently tagged secondary antibodies. BrdU is a thymidine analog that incorporates into dividing cells during DNA synthesis. Once incorporated into the new DNA, BrdU will remain in place and be passed down to daughter cells following division. Typically, BrdU is injected intraperitoneally. Different survival times required by the desired experimental time-line will yield data on specific phases of neurogenesis: proliferation, differentiation and maturation. One of the drawbacks of using BrdU is the dependence on a stressful injection procedure and uncertain penetration of the targeted cells with a uniform concentration of the compound. Thus, for experiments requiring measurements of cell proliferation, Ki67 can be used as an acceptable alternative. The protocol takes 3–5 d, allowing for sectioning and staining.

INTRODUCTION

The most important step to consider prior to the BrdU staining procedure is the experimental protocol used for BrdU administration. A common method is to inject BrdU intraperitoneally, but it can also be given in drinking water or injected locally into the brain ventricles. The dosages and the number of injections will depend on experimental design: 50–300 mg kg⁻¹ per injection is often used^{1,2}. BrdU is a 'gold standard' by which all other markers in neurogenesis research are measured^{3–6}. However, by itself, BrdU does not give any indication of the phenotype of marked cells.

Ki67 (discovered at The University of Kiel, hence the abbreviation) is a popular, alternative marker of cell proliferation. Ki67 is a large nuclear protein with a molecular mass of approximately 395 K; it is endogenously and differentially expressed at different levels during different phases of the cell cycle. Ki67 is expressed in dividing cells for the most of their mitotic process. It is not expressed during the resting phase (G₀) and during the early G1 phase of the cell cycle. Its expression begins at the onset of late G1 and ends once the cell exits the cycle and is in the G₀ phase⁷.

Ki67 undergoes phosphorylation and dephosphorylation during mitosis, it is susceptible to proteases and its structure implies that its expression is regulated by proteolytic pathways^{7–12}. Ki67 also shares structural similarities with other proteins that are known to be involved in cell-cycle regulation⁸. It has complex and specific localization patterns within the nucleus, which change during the cell cycle. The amount of Ki67 protein present during the cell cycle is highly regulated, presumably by precise synthesis and degradation systems, perhaps involving proteasome (a protease complex that has been shown to be involved in the degradation of Ki67)¹².

Similarly to BrdU, Ki67 can be detected using immunohistochemistry. Unlike BrdU, Ki67 is an endogenous marker that does not have any adverse effects on living cells. Although the function of Ki67 is not known, it is a reliable marker of mitosis due to its expression, albeit at different levels, during mitosis. The half-life of Ki67 is short and is not detectable during DNA-repair processes and its expression is confined to the nucleus¹¹. Moreover, studies reported so far show that Ki67 is expressed during mitosis in all mammalian species, from rodents to humans^{8,10}.

Doublecortin (DCX) is a marker of developing, immature neurons. DCX is a protein that is required for normal neuronal migration in the developing cerebral cortex¹³. DCX is a microtubule-associated phosphoprotein and it is selectively located in the periphery of the soma and its processes. Thus, DCX overlaps with microtubule distribution¹⁴.

DCX expression has been found both in the adult rostral migratory stream and in the dentate gyrus of the hippocampus of adult rats in the early differentiation stage of adult neurogenesis¹⁵. DCX can be used to identify young, immature neurons. Studies have shown that DCX expression in neurons is dependent on time and is typically expressed for the first 2 weeks after the birth of the neuron¹⁶. DCX labels the cytoplasm but not the nucleus, which makes it ideal for double labeling with the mitotic markers, BrdU and Ki67.

There are two notable alternatives to DCX. Collapsin response mediator protein 4 (CRMP-4)¹⁷ and polysialic-acid neural cell-adhesion molecule (PSA-NCAM)¹⁸. Both are expressed in parallel with DCX. PSA-NCAM is a particularly good alternative due to a large body of literature on its functional roles in cell development. This is in contrast to CRMP-4 (in mice, known as the mouse protein Ulip-1), the function of which is not well understood. However, there are many varieties of primary antibodies for CRMP-4 and PSA-NCAM (antibodies raised to various fragments of the target proteins), which originate from a number of laboratories, so careful planning is required when applying them to a new project. Moreover, PSA-NCAM can be expressed in glial cells, as well as in neuronal cells, making its use as a young neuronal marker ambiguous.

Most projects involving neurogenesis will also include markers of mature neurons. Two good choices are neuronal nucleus protein (NeuN)¹⁹ and calbindin (CaBP)^{20,21}. NeuN begins to be expressed within the first few days of cell development and persists in mature neurons²². NeuN is primarily located in the neuronal nuclei, but notably also stains the mossy fiber terminals of the granule neurons. CaBP is a cytoplasmic protein, and is presumably involved in calcium buffering. Its expression begins in 2–3-week-old neurons and normally persists during maturation¹⁶.

It should be noted, however, that all endogenous markers are labile and are influenced by cell activity. NeuN, for example, apparently disappears from hippocampal pyramidal neurons after ischemia²³, and CaBP is known to disappear from the dentate granule neurons after seizures²⁴. Furthermore, the developmental periods of marker expression specified here apply to laboratory rats (primarily Sprague–Dawley), but are different for mice and could also be different for other species.

Experimental time-line

Experimental design should begin with a clear time-line of the study, particularly when administration of BrdU is involved. The choice of markers will be largely dictated by the time-line. Measurements of proliferation, differentiation, maturation and survival require different time-lines and specific markers.

Tissue fixation

Tissue fixation and processing should be carried out expeditiously, although the fixed brain specimens can be kept for months in the fridge in phosphate-buffered saline (PBS) with 0.1% sodium azide without a significant loss of antigenicity. In cases in which such long-term storage is required, it is better to keep the brains or portions of the brains intact rather than in sections. The procedures listed below apply to the formaldehyde-fixed tissue. This is best done by intracardiac perfusion, but staining can also be performed on tissues that are treated differently. For example, tissue can be perfused with saline to clear blood vessels and post-fixed by submersion²⁵. In cases in which perfusion is not feasible (e.g., in natural species caught in the wild or in human brain tissue obtained from a clinician), adequate results can be obtained by post-mortem submersion of the tissue without prior perfusion¹⁰. In the latter case, dividing the tissue into small pieces will aid the fixation.

Tissue sampling

The whole structure of interest (e.g., hippocampus) needs to be sectioned, and all sections, even those that were damaged, should be kept in multiwell plates. Rat hippocampus, for example, can be sectioned in 30 µm sections and all sections kept in a single 48-well plate, allowing several sections per well. Sections in each well should be submerged in PBS with sodium azide.

Only a small number of sections from each hippocampus are generally sufficient to obtain a quantitative estimate of the cell

number, but each study will need a preliminary procedure to determine an optimal sampling scheme. The cardinal rule of sampling is that the variability of cell counts among the individual sections should be smaller than the variability among animals. In the case of the BrdU or Ki67 protocols, several stained cells per section are usually found; therefore, sampling of approximately 10–20 random sections per hippocampus is sufficient. This has to be estimated empirically, as explained by West *et al.*²⁶. In the case of DCX, there are usually 80–100 cells per section and fewer sections per hippocampus are sufficient.

Staining

Staining usually takes place one marker at a time, so separate successive incubations must be planned for double- or triple-marker labeling. A single primary antibody incubation usually takes 24–48 h and is followed by a secondary (2 h) incubation. It is advisable to check whether or not the first label works before proceeding with the double label on the same sections. The antigen unmasking, if required, precedes the primary antibody incubation and can add a few hours to the procedure.

Cell counting

Exhaustive counting of the stained cells, assuming 10 sections per animal and 5 animals per experimental group, will take several weeks.

Useful web resources

Potentially useful websites are:

- <http://www.biocompare.com/jump/2045/Antibody-Search.html>: A useful website to compare prices and to check the availability of most commercially available antibodies.
- <http://probes.invitrogen.com/resources/spectraviewer/>: A useful website to plot, compare and check the spectral compatibility of many fluorophores.
- <http://www.olympusmicro.com/>: A useful website with resources and tutorials designed to provide an Internet-based educational forum on all aspects of optical microscopy, photomicrography, confocal microscopy and digital imaging.
- <http://www.newneuron.com/>: An author's lab website with more examples of staining procedures, including an alternate description of the immunohistochemical technique.

MATERIALS

REAGENTS

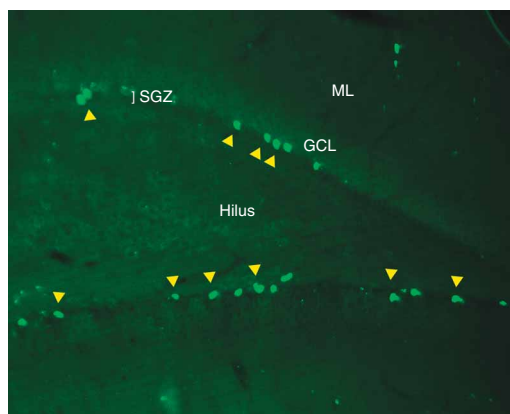
- Experimental animals: The rate of neurogenesis varies among species and can vary in different strains of the same species. The procedures described here work in laboratory rats (males: Sprague–Dawley, Long Evans) and in mice (males and females: C57B6). The rate of neurogenesis is strongly age-dependent. A 1-year-old rat has only about a 5–10% rate of neurogenesis of a 1-month-old rat. Although some studies still give animal weights to indicate the approximate age, we strongly recommend tracking the exact age as the weight can vary with the animal's housing conditions.
- Positive controls: Sample brains from young animals injected with BrdU should be kept as positive controls. These can be stored in the fridge for up to 1 year. Hippocampal sections from such controls inevitably express all markers that have been discussed in this protocol (see Figs. 1–5).

! CAUTION All experiments are to be performed in accordance with relevant authorities' guidelines and regulations.

- Inhalational anesthetic, isoflurane (Abbott Laboratories Ltd.)
 - Sodium phosphate, monobasic anhydrous NaH₂PO₄ (Sigma, cat. no. S-0751; 1 kg, FW 120.0)
 - Sodium phosphate, dibasic anhydrous Na₂HPO₄ (Sigma, cat. no. S-0876; 1 kg, FW 142.0)
 - Normal serum (goat; for DCX staining, use horse)
 - Triton X-100 (t-Octylphenoxypolyethoxyethanol; Sigma, cat. no. T-9284; 100 ml)
 - Tri-sodium citrate dihydrate (Sigma, cat. no. W302600; FW 294.10)
 - Paraformaldehyde powder (Sigma, cat. no. P6148; 1 kg)
 - BrdU ((+)-5'-Bromo-2'-deoxyuridine; 97%; Sigma-Aldrich, cat. no. 858811)
- ! CAUTION** Paraformaldehyde powder is harmful and BrdU is a suspected mutagen when administered to prenatal or neonatal animals^{27,28} and should be handled with care. Wear proper protective gear, a lab coat and weigh under a fume hood.



Figure 1 | BrdU-positive cells in a coronal section (rat, male, Sprague-Dawley, 35–40 d old, 10× objective) of the dentate gyrus. BrdU was injected intraperitoneally three times at 4-h intervals at 33.3 mg kg⁻¹. The brain was perfused 1 week following the last injection. Yellow arrows point to labeled cells in the subgranular zone (SGZ). GCL, granule cell layer; ML, molecular layer.



- Rat anti-BrdU primary monoclonal antibody (clone BU1/75, ICRI; Accurate Chemical & Scientific, cat. no. OBT0030); optimal dilution ratio 1:200
- Rabbit anti-human Ki67 polyclonal primary antibody (Vector Laboratories, cat. no. VP-K451); optimal dilution 1:200
- Mouse anti-human Ki67 monoclonal primary antibody (clone MIB-1; DakoCytomation, cat. no. M7240); optimal dilution 1:100
- Goat anti-DCX polyclonal primary antibody (C-18; Santa Cruz Biotechnology, cat. no. SC-8066); optimal dilution ratio 1:200
- Alexa Fluor 488 chicken anti-Rat IgG (H+L) secondary antibody (Molecular Probes); optimal dilution ratio 1:200
- Alexa Fluor 568 goat anti-rabbit/mouse IgG (H+L) secondary antibody (Molecular Probes); optimal dilution ratio 1:200
- Alexa Fluor 594 donkey anti-Goat IgG (H+L) (Molecular Probes); optimal dilution ratio 1:200

EQUIPMENT

- Dissecting tools
- Syringes
- 23–27G 1-inch needle, preferably with a short bevel
- Perfusion pump or 50-ml syringe
- Glass vials or 15-ml polypropylene conical tubes, 17 × 120 mm (Becton Dickinson)
- Vibratome/Microtome/Cryostat
- 24- or 6-multiwell tissue cell culture polystyrene plates
- Oven (37–45 °C)
- Shaker
- Microscope slides (VWR, cat. no. 48323-185; 76 × 26 mm)
- Micro cover-glasses (VWR, cat. no. 48404 454; 24 × 60 mm)
- Fluorescent microscope
- Confocal microscope

REAGENT SETUP

BrdU injection solution Warm saline solution (0.9% w/v NaCl in sterile H₂O) to 40–50 °C, slowly dissolve BrdU in saline solution by gently vortexing, allow the BrdU injection solution to cool to room temperature (22–25 °C) and use the BrdU injection solution immediately.

Storage at 4 °C is not recommended due to the formation of white BrdU precipitates (crystals). A 20 mg ml⁻¹ stock is good for most cases that require intraperitoneal injections. **▲ CRITICAL** White BrdU precipitates can be re-dissolved at 40–50 °C for later use. The solubility of BrdU in normal saline is pH-dependent and BrdU dissolved in saline is acidic; therefore, the addition

of NaOH (0.01 M) may be required to keep the pH as neutral as possible. However, a basic solution may cause an adverse reaction with the animals' skin and tissue; therefore, heating and slowly dissolving BrdU while titrating the pH towards 7 without making the solution basic is preferred.

! CAUTION There is some evidence in the literature that BrdU may be carcinogenic and produce developmental abnormalities when given to prenatal and neonatal animals^{27,28}. The compound should be handled in the fume hood. The solution of BrdU should be handled with gloves.

PBS, 0.1 M, pH 7.4 In a 1–2 L beaker, add 2.7 g of sodium phosphate monobasic NaH₂PO₄, 11.5 g of sodium phosphate dibasic Na₂HPO₄ and 9 g NaCl. Add distilled water up to 1000 ml and stir. Measure the pH, which should be around 7.4.

Blocking solution, 0.1 M PBS, 0.3% Triton X-100, 2% serum In a 1–2 L beaker, add 20 ml of serum, 3 ml of Triton X-100, 0.1 M PBS up to 1,000 ml and stir. Store in 50-ml aliquots at –20 °C. (Ideally, the serum should be taken from the species of animal in which the secondary antibodies are made. Alternatively, normal horse serum can be used instead of goat serum.)

Sodium citrate buffer, 10 mM, 0.05 Triton X-100, pH 6 In a 1–2 L beaker, add 2.94 g of tri-sodium citrate (dihydrate) and distilled water up to 1,000 ml and stir. Adjust pH to 6.0 with 1 M HCl. Add 0.5 ml of Triton X-100. Store at 4 °C for up to 6 months.

4% Paraformaldehyde in 0.1 M PBS In a 1–2 L beaker, add about 800 ml of 0.1 M PBS. Heat 0.1 M PBS to 60–65 °C while stirring. At 60–65 °C, add 40 g of paraformaldehyde powder slowly while stirring (note: adding a few drops of 1 M NaOH helps to keep the solution clear). Continue to stir until the paraformaldehyde powder is dissolved, making sure that the temperature stays between 60 and 65 °C. Cool the solution until it reaches room temperature. Filter solution and keep at 4 °C. **! CAUTION** Prepare in the fumehood and keep in the fridge until use. Can be kept for several weeks.

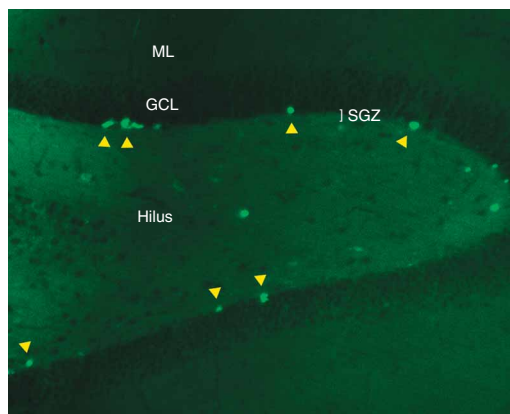


Figure 2 | Ki67-positive cells in a coronal section (rat, male, Sprague-Dawley, 35–40 d old, 10× objective) of the dentate gyrus. Yellow arrows point to labeled cells in the subgranular zone (SGZ). GCL, granule cell layer; ML, molecular layer.

PROCEDURE

BrdU injection

1| Inject BrdU solution intraperitoneally.

▲ CRITICAL STEP The ideal BrdU injection dosage varies from 50 mg kg⁻¹ to 300 mg kg⁻¹ depending on the experiment and the animal in use. Multiple 50 mg kg⁻¹ injections are often used to ensure the labeling of many cells undergoing cell divisions over a period of time, usually several days (usually in mice). This procedure is adequate when the precise dating of cells is not required. Higher doses in the order of 300 mg kg⁻¹ are preferred when the time-line of the experiment is short and the age of the cells is of essence. The higher dose also ensures that the BrdU is not 'diluted' in the process of re-division of daughter cells^{1,16,22}. Using the stock solution described above, approximately 5 ml of BrdU solution is required for a 500 g adult rat to obtain 200 mg kg⁻¹.



PROTOCOL

■ **PAUSE POINT** For an intraperitoneal injection, the lower abdominal cavity must be isolated. In mice, we use a 27-gauge needle and a 1-ml syringe. The maximum intraperitoneal injection volume in the mouse is 3 ml. For rats, we use a 23-gauge needle and a 5-ml syringe. The maximum tolerable intraperitoneal injection volume in the rat is 10 ml.

Anesthesia

2| Animals are first anesthetized with isoflurane. Apply approximately 1 ml of isoflurane to tissue paper and place it in a closed chamber. Immediately place the animals in the chamber and close the lid and wait for 1 min.

▲ **CRITICAL STEP** Isoflurane is a health hazard, so it should be handled with care. Proper protection, such as a lab coat, mask and goggles should be worn at all times. Moreover, as isoflurane is an inhalation anesthetic, all work involving isoflurane should be conducted under a properly ventilated fume hood.

3| After 1 min, monitor and test the animal to determine whether the animal is fully anesthetized and ready for transcardial perfusion. Fully anesthetized animals should display the following properties: respiratory rate (breathing) should be regular and relaxed; withdrawal reflexes should be absent (this can be tested by pinching one of the paws); and there should be no response to external stimuli (e.g., blowing air on the eye). If the animal still displays signs of awareness, place the animal back into the chamber for 1 more minute and repeat the monitoring and testing.

Transcardial perfusion

4| Expose the heart using sharp dissecting tools.

5| Insert the needle connected to the pump into the left ventricle. Make an incision in the right atrium to allow blood to flow out of the animal's body.

6| Perfuse the animal with PBS, 0.1 M at pH 7.4.

7| When the draining blood becomes clear, perfuse the animal with 4% paraformaldehyde in 0.1 M PBS.

▲ **CRITICAL STEP** The amount of 4% paraformaldehyde in 0.1 M PBS required for perfusion varies with the weight of the animal. Typically, a rat weighing 500 g will require 200 ml solution.

Dissection

8| Using appropriate dissecting tools, remove the head, then remove the muscle and membranous tissue from the top part of the skull and gently extract the brain from the skull.

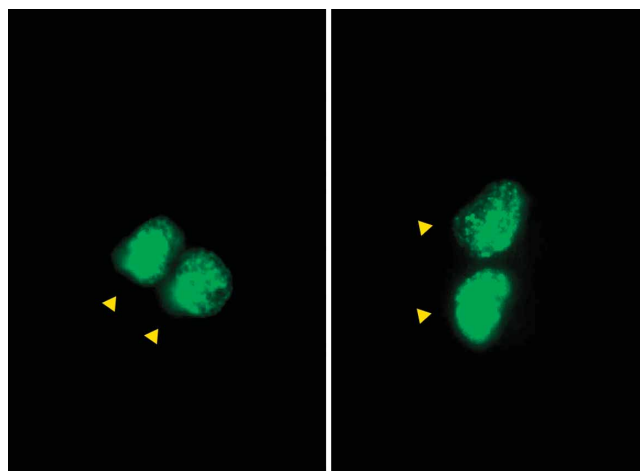


Figure 3 | Examples of BrdU (left) and Ki67 (right) positive nuclei shown at high magnification (100×). Images were taken 24 h after BrdU injection. (Obtained from Kee *et al.*, 2002 with permission from Elsevier.)

Dissection

8| Using appropriate dissecting tools, remove the head, then remove the muscle and membranous tissue from the top part of the skull and gently extract the brain from the skull.

9| Cut the trigeminal and optic nerves, and let the brain fall into a beaker of cold 4% paraformaldehyde in 0.1 M PBS.

Post-fixation

10| Immerse the removed brain in the 4% paraformaldehyde in 0.1 M PBS for 24–48 h.

■ **PAUSE POINT** Tissues can be left for 1–2 d in the 4% paraformaldehyde in 0.1 M PBS at 4 °C.

▲ **CRITICAL STEP** Over-fixation may result in a lack of staining due to the unavailability of antigens.

Sectioning

11| Use the vibratome to section tissue into 10–40 μm slices.

▲ **CRITICAL STEP** Vibratome sections have some advantages when performing immunohistochemistry as the tissue is not processed further and keeps the antigenicity better than some other methods (e.g., paraffin embedding, ethanol fixing). Vibratome sections allow the morphology of tissue sections to be

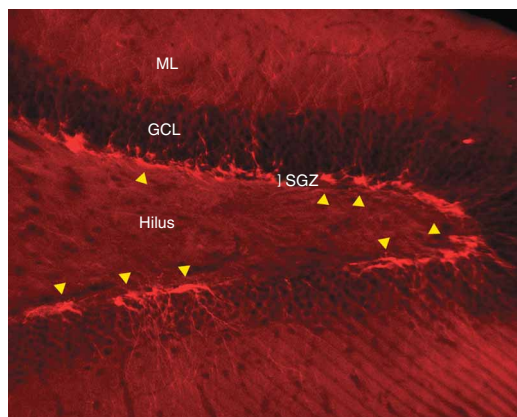


Figure 4 | Doublecortin-positive cells in a coronal section (rat, male, Sprague–Dawley, 35–40 d old, 10× objective). Yellow arrows point to labeled cells found in the inner granule cell layer (GCL). ML, molecular layer; SGZ, subgranular zone.

un-disrupted due to no freezing and thawing steps. The disadvantage of vibratome sections is the slow and difficult sectioning step. In poorly fixed tissues, the formation of vibration marks or vibratome lines, which are often visible, may hinder analysis in the sections.

DNA denaturation (required only for BrdU immunohistochemistry; otherwise, go to Step 16)

12| Transfer sections to 24-well plates loaded with 0.1 M PBS (at pH 7.4).

13| Rinse sections three times, 5 min each with 0.1 M PBS (at pH 7.4) on a shaker.

14| Denature DNA by incubating sections in 1 M HCl for 30 min at 45 °C (or 2 M HCl for 15 min at 37 °C).

▲ **CRITICAL STEP** Denaturation of DNA allows access for the anti-BrdU antibody so incomplete denaturation causes problems.

There are various denaturation procedures (such as ethanol treatment and enzyme treatment), which may have different effects on the retention of morphology. The acid treatment with an increased temperature generally results in more effective exposure of the halogenated-nucleotide antigen. However, harsh HCl treatment (> 2 M HCl), in conjunction with high temperature (> 65 °C) incubation, is detrimental to other antigens, particularly surface antigens and receptors. Moreover, some BrdU antibodies can recognize methylated thymidine under harsh denaturation conditions. This non-specific staining is evident when all or most of the nuclei are stained. Thus, careful adjustments of the denaturation conditions are necessary.

■ **PAUSE POINT** Earlier protocols have used a pre-treatment step using incubation with formamide at 65 °C, which precedes the HCl step; however, with the advent of new BrdU antibodies, this step is not required (see **Figs. 1,3**).

15| Neutralize the acid by rinsing sections three times, 5 min each with 0.1 M PBS (at pH 7.4) on a shaker.

Antigen retrieval (required for use with monoclonal Ki67 primary antibodies; otherwise, go to Step 21)

16| Rinse sections three times, 5 min each in 0.1 M PBS (at pH 7.4).

17| Transfer the sections to 10 mM sodium citrate buffer (at pH 6), preheated to 80 °C in a water bath.

18| Keep sections in 10 mM sodium citrate buffer (at pH 6) at 80 °C for 30 min.

19| Keep sections in 10 mM sodium citrate buffer (at pH 6) while allowing the sections to cool to room temperature.

20| Rinse sections three times, 5 min each in 0.1 M PBS (at pH 7.4).

BrdU, Ki67, DCX single immunohistochemistry

21| Transfer sections to 24-well plates loaded with 0.1 M PBS (at pH 7.4).

22| Rinse sections three times, 5 min each in 0.1 M PBS (at pH 7.4) on a shaker.

23| Incubate sections with blocking solution for 60 min at room temperature on a shaker.

24| Incubate sections with BrdU or Ki67 and/or DCX primary antibody diluted in blocking solution for 24–48 h at 4 °C on a shaker.

■ **PAUSE POINT** Tissues can be left overnight at 4 °C or longer.

25| Rinse sections three times, 5 min each in 0.1 M PBS (at pH 7.4) on a shaker.

26| Incubate sections with fluorochrome-conjugated secondary antibody in the dark, diluted in 0.1 M PBS (at pH 7.4) with 0.3% Triton X solution (detergent such as Triton-X can be added to the secondary antibody diluent to reduce hydrophobic interactions between tissue and reagent proteins, thus reducing non-specific binding of secondary antibodies) for 2 h at room temperature on a shaker.

27| Rinse sections three times, 5 min each in 0.1 M PBS (at pH 7.4) on a shaker.

28| If you are performing a single immunohistochemistry analysis only, go to Step 31 (and see **Figs. 1–5**).

Second immunolabeling for BrdU/DCX double immunohistochemistry

29| After following instructions from Steps 12–27 using BrdU primary antibody, proceed to Step 30.

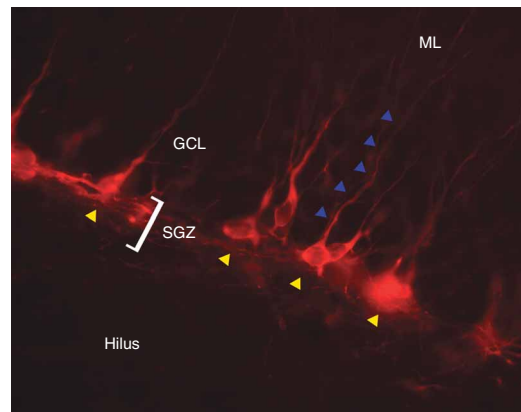


Figure 5 | Doublecortin-positive cells. As in **Figure 4**, but at higher magnification (40× objective) to illustrate the morphology of the immature neurons. GCL, granule cell layer; ML, molecular layer; SGZ, subgranular zone.

30| Repeat the instructions from Steps 12–27 with the second primary antibody against DCX; then continue to Steps 31–32.

▲ CRITICAL STEP Double or triple-color immunostaining is complicated. It is important to choose antibodies that are compatible with the fixation, embedding and that will not cross-react. Performing single-color staining of the BrdU and the other markers (e.g., DCX, NeuN and CaBP D28K) prior to and in parallel with the double-color staining can be helpful. Once the optimal conditions for BrdU and DCX have been determined (titration), we recommend sequential reactions (BrdU staining first and DCX staining thereafter) for double immunostaining. The sequential reactions for double immunostaining have two main advantages over the simultaneous (addition of the BrdU and DCX antibodies together in a cocktail) double immunostaining reaction. First, the likelihood of cross-reaction between the secondary antibodies is reduced. For example, the order in which the secondary antibodies are added can be controlled to avoid non-specific binding (e.g., when using goat anti-mouse and donkey anti-goat secondary antibodies, donkey-anti-goat antibody reaction can be performed before the addition of goat anti-mouse to avoid non-specific binding). Second, each staining step can be verified before continuing onto the next step. Thus, for novices, this is a good way to troubleshoot in case of problems. In experienced hands, the sequential reactions can be performed simultaneously. In the latter case, a simultaneous double immunostaining reaction with properly chosen secondary antibodies (the secondary antibodies should ideally come from the same host to avoid cross-reaction) works well and saves time.

31| Carefully transfer sections to slides using a soft brush.

32| Add mounting medium (Permafluor) and place coverslips.

■ PAUSE POINT Mounted tissues can be kept at 4 °C for up to 6 months before imaging with a microscope.

? TROUBLESHOOTING

See **Table 1** for troubleshooting advice.

TABLE 1 | Troubleshooting table.

Problem	Possible reason	Action
BrdU/Ki67/DCX: Poor positive staining and/or no positive staining with little or no background staining.	BrdU/Ki67/DCX primary and secondary antibody concentration was not optimal.	Titrate BrdU/Ki67/DCX primary and secondary antibody when it arrives, as concentrations may vary across batches. This should be used with the tissue that serves as positive control.
	Incubation time with primary and/or secondary antibody was too short.	Use longer incubation times for primary antibody and/or secondary antibodies.
	The primary antibody does not recognize the antigen due to incorrect or over-fixation.	Include antigen retrieval step. Note: For BrdU primary antibody, over-fixation does not effect BrdU immunostaining.
	Antigen destroyed by excessive antigen retrieval step.	Reduce antigen retrieval time.
BrdU: Poor positive staining and/or no positive staining with little or no background staining.	Slides were left to dry.	Do not let slides dry out and keep wet at all times during the staining procedure.
	Denaturation of DNA by HCl was not sufficient.	Use higher concentration of HCl and/or longer incubation time. In addition, use increased temperature with HCl incubation.
Ki67: Poor positive staining and/or no positive staining with little or no background staining.	Antigen retrieval time was too short so that the Ki67 antigen was not available to Ki67 primary antibody.	Increase antigen retrieval time.
BrdU/Ki67/DCX: Non-specific and/or high background staining.	Non-specific binding of primary or secondary antibody.	Increase the number and time of washes in between steps.
	Incubation time with BrdU/Ki67/DCX primary antibody was too long.	Reduce BrdU/Ki67/DCX primary antibody incubation time.
	Blocking reaction was not optimal to allow for non-specific binding of the secondary antibodies.	Increase length and/or concentration of incubation with blocking solution made from the same species as the host of the secondary antibody.
	Aggregates binding.	Centrifuge antibody stock briefly in a micro-centrifuge at high speed to remove aggregates.



● TIMING

- Steps 1–11: tissue preparation, 2 d, including perfusion, overnight post-fixation and cutting
- Steps 12–15: DNA denaturation, 90 min
- Steps 16–20: antigen retrieval, 90 min
- Steps 22–23: blocking, 90 min
- Step 24: primary antibody, 24–48 h
- Steps 25–27: secondary antibody, 180 min

ANTICIPATED RESULTS

The pilot experiment evaluates the extent of neurogenesis in young rats using three principal markers: BrdU, Ki67 and DCX (see **Figs. 1–5**). By injecting three times on a given day, we are likely to label most of the cells dividing on that day. By perfusing 1 week after the injections, we expect to see the surviving cells from the cohort that divided 1 week previously. In a selected section, we observed more than 15 labeled nuclei in the subgranular zone. This is more than the usual number seen per section. Typically, a few additional nuclei were located in the Hilus and the molecular layer.

Ki67 labeling was used to verify the rates of proliferation at the time of the perfusion. The location and appearance of the nuclei were similar to those labeled with BrdU, although there was also a noticeable presence of clusters of nuclei that were not yet dispersed. DCX shows young neurons that are likely to be 1–2 weeks old. The ‘patchy’ distribution of these cells along the subgranular zone is typical. Some are sending dendrites towards the molecular layer and most are sending slender axons towards the hilus. Overall, the experiment shows robust neurogenesis in the young rat.

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COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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