Video Article

Immunohistochemistry and Multiple Labeling with Antibodies from the Same Host Species to Study Adult Hippocampal Neurogenesis

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Abstract

Adult neurogenesis is a highly regulated, multi-stage process in which new neurons are generated from an activated neural stem cell via increasingly committed intermediate progenitor subtypes. Each of these subtypes expresses a set of specific molecular markers that, together with specific morphological criteria, can be used for their identification. Typically, immunofluorescent techniques are applied involving subtype-specific antibodies in combination with exo- or endogenous proliferation markers. We herein describe immunolabeling methods for the detection and quantification of all stages of adult hippocampal neurogenesis. These comprise the application of thymidine analogs, transcardial perfusion, tissue processing, heat-induced epitope retrieval, ABC immunohistochemistry, multiple indirect immunofluorescence, confocal microscopy and cell quantification. Furthermore we present a sequential multiple immunofluorescence protocol which circumvents problems usually arising from the need of using primary antibodies raised in the same host species. It allows an accurate identification of all hippocampal progenitor subtypes together with a proliferation marker within a single section. These techniques are a powerful tool to study the regulation of different progenitor subtypes in parallel, their involvement in brain pathologies and their role in specific brain functions.

Video Link

The video component of this article can be found at https://www.jove.com/video/52551/

Introduction

Two brain regions constitutively generate new neurons throughout life, the subventricular zone of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG). The newborn neurons derive from neural progenitor cells and go through different stages of morphological and physiological development before reaching maturity[1,2]. From a slowly dividing radial glia-like stem cell (type 1) consecutive stages of transit amplifying intermediate progenitor cells arise. The more undifferentiated subtypes (type 2a and type 2b) have an irregular shape with short, tangential processes. They generate neuroblasts (type 3) that gradually exit the cell cycle to become immature neurons (with dendrites extended towards the molecular layer) and finally integrate into the hippocampal network as mature granule cells. Due to their particular physiological characteristics these cells provide the circuitry with enhanced plasticity[3] suggesting a unique role in hippocampal function. Actually, studies of the last decade generated substantial evidence that adult neurogenesis contributes to spatial memory, pattern separation and emotional behavior[4,5].

Adult neurogenesis can be studied using different approaches. Thymidine analogs incorporate into DNA during the S-phase of the cell cycle and allow birth dating, quantification and fate analysis of newborn cells[6-8]. Sequential application of different thymidine analogs (*e.g.,* CldU, EdU or IdU) can be used to study cell turnover or cell populations born at different time points during the course of an experiment[9]. An alternative, endogenous marker for cell proliferation is Ki67. It is expressed in dividing cells during all phases of the cell cycle (G1, S, G2, M) except the resting phase (G0) and the beginning of G1 [10,11]. To analyze the phenotype of newborn cell populations in the adult dentate gyrus several stage-specific molecular markers can be used such as GFAP, nestin, DCX and NeuN[1,6]. GFAP is a marker of mature astrocytes but is also expressed in radial glia-like cells in the adult forebrain. Nestin is an intermediate filament specific for radial glia-like cells and early intermediate progenitor cells. DCX is a microtubule-associated protein expressed in intermediate progenitors, neuroblasts and immature neurons. Based on the (co-) expression of these three markers and the morphological features of the labeled cells four distinct progenitor cell subtypes can be identified: type 1 ($GFAP^+$, $nestin^+$, DCX^-), type 2a ($GFAP^-$, $nestin^+$, DCX^-), type 2b ($GFAP^-$, $nestin^+$, DCX^+) and type 3 ($GFAP^-$,$nestin^-$, DCX^+)[1]. Co-labeling of DCX together with NeuN, which is expressed in postmitotic neurons, allows the differentiation of immature (DCX^+, $NeuN^+$) and mature (DCX^-, $NeuN^+$) granule neurons.

The above mentioned markers are frequently used for immunofluorescent co-labeling and subsequent confocal microscopy to analyze the number and identity of newborn cells. This typically requires antibodies from different host species to prevent undesired antibody cross-reactivity. However, the majority of primary antibodies suitable for neurogenesis research are raised either in rabbits or mice (*e.g.,* mouse α-

BrdU, mouse α-NeuN, rabbit α-Ki67, rabbit α-GFAP). This leads to serious limitations in the number and combination of antigens that could be evaluated in a single slice. This in turn not only increases the staining effort, as multiple stainings have to be performed, but might also compromise the reliability of results. Furthermore, some antigens are susceptible to formalin fixation-induced epitope masking (*e.g.,* Ki67, nestin). We herein describe modifications from the classical single- and multiple immunolabeling protocols (*e.g.,* epitope retrieval, multiple sequential immunostaining, use of nestin-GFP transgenic mice[12]) that overcome many of these issues. In particular, the sequential multiple immunofluorescence protocol allows staining against up to four different antigens even if part of the antibodies is derived from the same host. This enables the simultaneous detection of type 1, type 2a, type 2b and type 3 progenitor cells, as well as their proliferative activity within a single section.

Protocol

NOTE: All procedures involving living animals were carried out in accordance with the EC directive 86/609/EEC guidelines on the care and use of laboratory animals and approved by the local ethics committee (Thüringer Landesamt für Lebensmittelsicherheit und Verbraucherschutz).

1. Intraperitoneal Injection of Thymidine Analogs

1. Weigh animals the day before injection. Calculate the amount of thymidine analog required for all injections planned on the next day as well as individual weight-adjusted injection volumes of a 10 mg/ml stock solution.
2. Prepare 10 mg/ml thymidine stock solution. (**CAUTION!** Thymidine analogs are toxic. Follow the specific Material Safety Data Sheets (MSDS) provided by the suppliers, *i.e.,* wear lab coat and gloves, use a chemical fume hood).
    1. Take thymidine analog from the freezer and bring it to RT, approx. 21 °C. Weigh 10 mg and add sterile saline (for BrdU and CldU) or 0.04 N NaOH (in sterile saline; for IdU), vortex. Place for at least 10 - 15 min in a 50 °C water bath and vortex every 2 - 3 min to dissolve the powder.
    NOTE: Use solutions for up to 24 hr when stored at RT and for several weeks at -20 °C. Protect solutions from light (cover with aluminum foil). Always check for precipitates and re-dissolve if necessary.
3. Restrain the mouse by the scruff and intraperitoneally inject an appropriate, weight-adjusted volume of stock solution (at RT) using a fine dosage syringe with a 30 G needle.
NOTE: In case CldU and IdU have to be administered sequentially within the same animal, consider to inject equimolar concentrations (*e.g.,* 42.5 mg/kg CldU and 57.5 mg/kg IdU, which correspond to 50 mg/kg BrdU). Therefore, adjust the injection volumes of the 10 mg/ml stock solutions accordingly.

2. Tissue Preparation

1. Prepare 4% formaldehyde in 0.1 M phosphate buffer pH 7.4 on the day before perfusion. Store at 4 °C.
2. Transcardially perfuse the deeply anesthetized mice (3.5% isoflurane) via the left ventricle with 10 ml ice-cold PBS, then with 40 ml ice-cold formaldehyde (flow rate 5 ml/min). Dissect the brain and post-fix in the same fixative for 24 hr at 4°C.
3. Transfer the brains consecutively into 10% (24 hr at 4°C) and 30% sucrose (until the brain sinks, approx. 48 hr). For freezing, slowly submerge the cryoprotected brains into -25 °C isopentane until no bubbles emerge from the tissue. Store at -80 °C.
4. Cut coronal sections of 40 µm thickness on a freezing microtome (block temperature at -25 to -16 °C). Sequentially transfer sections into antifreeze solution containing wells of a 24-well cell culture plate (see **Figure 1**). Store at -20 °C.

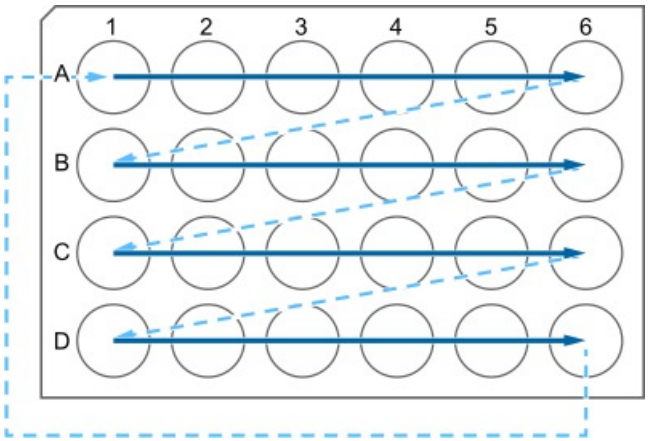

Figure 1. Schematic illustration of transferring microtome slices into a 24-well plate. Start at A1 and place subsequent slices into row A, after A6 go to the next row B and so forth. When reaching D6, go back to A1 and continue. This arrangement of slices allows for quantification of every n^{th} section of an entire brain. For quantification of newborn cells take every 6^{th} brain section (equivalent to the content of one column), for immunofluorescence phenotyping take every 12^{th} section (equivalent to the content of 2 alternating rows of one column).

3. Immunostaining

NOTE: Sections are processed free floating, usually in 6-well plates equipped with a carrier plate and mesh inserts. As an exception, blocking, antibody incubations and ABC reaction are done in 12- or 24-well plates without mesh inserts (0.5 to 1 ml per well is sufficient, depending on the number of slices that have to be stained). During these steps, transfer sections with the help of a fine brush (rinse with each new solution). All incubations are done with continuous agitation (max 150 rpm).

1. **Immunohistochemistry (ABC method)**
    1. Transfer sections from antifreeze into TBS and rinse thoroughly (once O/N at 4 °C, 5 times at RT for 10 min each) to completely remove antifreeze.
    2. Incubate for 30 min in 1.5% H_2O_2 in TBS-T to quench endogenous peroxidase activity. Pay attention to bubbling and re-submerge sections if necessary. Rinse 3 times in TBS for 15 min each.
    3. *Optional:* Meanwhile preheat a heating cabinet and 2 N HCl to 37 °C. Incubate sections for 30 min at 37 °C in 2 N HCl to denature DNA. Gently separate sections with the help of a brush.
    4. *Optional:* Neutralize sections for 10 min in 0.1 M borate buffer pH 8.5, RT. While transferring, briefly swab the mesh inserts containing the sections on a paper towel to remove HCl leavings. Rinse 2 times in TBS for 15 min each.
    5. Incubate in TBSplus to permeabilize the tissue and to block unspecific antibody binding sites, 1 hr at RT.
    6. Incubate in primary antibody diluted in TBSplus, O/N at 4 °C. Rinse 3 times in TBS for 15 min each.
    7. Incubate in biotinylated secondary antibody diluted in TBSplus, 3 hr at RT. Rinse 3 times in TBS for 15 min each. Meanwhile...
    8. Prepare ABC complex according to manufacturer's protocol (1% A + 1% B in TBS-T). Allow to stand for 30 min at RT before use. Incubate sections in AB reagent for 1 hr at RT. Rinse 3 times in TBS for 15 min each.
    9. Prepare 50 ml 0.5 mg/ml DAB in TBS-T per 6- or 12-well plate, split into two halves and pipette 4 ml or 2 ml per well, respectively. Transfer sections into DAB solution (**CAUTION!** DAB is toxic. Follow the specific MSDS provided by the supplier, *i.e.,* wear lab coat and gloves, use a chemical fume hood).
    10. Add 0.5 ml 1% H_2O_2 to the remaining 25 ml DAB solution, mix and pipette equivalent volumes as above to each well to start peroxidase reaction. Incubate for 12 min. Rinse 3 times in TBS for 15 min each.
    11. Mount sections to slides in gelatin, air dry O/N. Coverslip with permanent mounting medium.
    12. *Optional:* Counterstain before placing the coverslip (see section 3.5).
        NOTE: If the signal-to-noise ratio is low because of high background, repeat H_2O_2 treatment after the incubation with AB reagent (step 3.1.8).

2. **Multiple-immunofluorescence**
    1. *Single or simultaneous multiple immunofluorescence*
        1. Transfer sections from antifreeze into TBS and rinse thoroughly (once O/N at 4 °C, 5 times at RT for 10 min each) to completely remove antifreeze.
        2. *Optional:* as steps 3.1.3 - 3.1.4.
        3. Incubate in TBSplus to permeabilize the tissue and block unspecific antibody binding sites, 1 hr at RT.
        4. Incubate in primary antibody cocktail (*e.g.,* rat α-BrdU, guinea pig α-DCX, goat α-GFP) diluted in TBSplus, O/N at 4°C. Rinse 3 times in TBS for 15 min each.
        5. Incubate in cocktail of fluorochrome-conjugated secondary antibodies (*e.g.,* Rhodamine Red α-rat, Alexa-647 α-guinea pig, Alexa-488 α-goat; all derived in donkey) diluted in TBSplus, 3 hr at RT or O/N at 4 °C. From now on protect sections from light. Rinse 3 times in TBS for 15 min each.
        6. Mount sections to slides in gelatin, air dry O/N. Coverslip with aqueous mounting medium.

    2. *Sequential multiple immunofluorescence with primary antibodies from same host species*
        1. As steps 3.2.1.1 - 3.2.1.3.
        2. Incubate in first primary antibody (*e.g.,* rabbit α-antigen A), O/N at 4 °C. Rinse 3 times in TBS and once in TBS-T for 10 min each.
        3. Incubate in first fluorochrome-conjugated secondary antibody (*e.g.,* Rhodamine Red-conj. donkey α-rabbit), 3 hr RT. From now on protect sections from light. Rinse 3 times in TBS and once in TBS-T for 10 min each.
        4. Incubate in 10% normal serum from same host as the primary antibodies (*e.g.,* rabbit serum) for 3 hr RT to saturate open paratopes on the first secondary antibody. Rinse 3 times in TBS and once in TBS-T for 10 min each.
        5. Incubate in TBSplus with 50 µg/ml unconjugated monovalent Fab fragments directed against the host of the primary antibodies (*e.g.,* α-rabbit IgG (H+L)) to cover epitopes that could be recognized by the second secondary antibody, O/N at 4 °C.
        6. Rinse at least 3 times in TBS and once in TBS-T for 10 min each. While transferring, briefly swab the mesh inserts containing the sections on a paper towel to remove any Fab leavings.
        7. Incubate in second primary antibody (*e.g.,* rabbit α-antigen B), O/N at 4 °C. Rinse 3 times in TBS and once in TBS-T for 10 min each.
        8. Incubate in second fluorochrome-conjugated secondary antibody (*e.g.,* Alexa-488-conj. donkey α-rabbit), 3 hr RT. Rinse 3 times in TBS for 15 min each, mount and coverslip as above.
            NOTE: To label antigens with antibodies from different host species add them to step 3.2.2.2 and the respective secondary antibodies to step 3.2.2.3.

    3. *One of the secondary antibodies from same species as one of the primary antibodies*
        NOTE: This protocol is suitable for quadruple-staining against BrdU or Ki67 together with GFAP, nestin-GFP and DCX.
        1. Follow strictly protocol steps 3.2.1.1 - 3.2.1.5. Until this point use only donkey serum in TBSplus.
        2. Incubate in TBSplus containing 3% goat serum for 1 hr at RT. This covers open paratopes on the α-goat secondary antibody. Rinse 3 times in TBS and once in TBS-T for 10 min each.

3. Incubate in AMCA-conj. goat α-rabbit diluted in TBSplus, 3 hr RT or O/N 4 °C. Rinse three times in TBS for 15 min each, mount and coverslip as above.

4. *CldU, IdU co-staining*
    1. Rinse, denature and neutralize sections as described in section 3.2.1 (steps 3.2.1.1 - 3.2.1.2).
    2. Incubate with 20 µg/ml unconjugated Fab fragments α-mouse IgG (H+L) in TBSplus, 1 hr at RT. Rinse 4 times in TBS and once in TBS-T for 10 min each.
    3. Incubate in primary antibody cocktail containing rat α-BrdU (1:400; purified IgG2) and mouse α-BrdU (1:350) diluted in TBSplus, O/N at 4°C. Rinse 3 times in TBS and once in TBS-T for 10 min each.
    4. Incubate in secondary antibody cocktail containing biotinylated donkey α-rat (1:500) and FITC-conj. donkey α-mouse Fab fragments (1:100). Rinse 3 times in TBS and once in TBS-T for 10 min each.
    5. Incubate in Rhodamine Red-conj. Streptavidin, 2 hr at RT. Rinse 3 times in TBS for 15 min each, mount and coverslip as above.

5. *Amplification of fluorescence signal in nestin-GFP mice*
    1. Add goat α-GFP to the primary antibody cocktail.
    2. Add a fluorochrome conjugated secondary antibody with spectral properties similar to GFP (such as Alexa 488 conj. donkey α-goat) to the secondary antibody cocktail.

3. **Epitope retrieval**
    1. Carry out epitope retrieval after rinsing out antifreeze. Preheat steamer with 6- or 12-well plates containing 0.1 M citrate buffer pH 6.0 to 95 - 99 °C (approximately 25 min).
    2. Transfer the sections into the hot citrate buffer and steam for 30 min (cover the plates with aluminum foil as plastic lids are not heat-resistant).
    3. Immediately place the plates in an ice bath to cool down. This helps to protect tissue morphology, which is of particular importance when working with brain sections of postnatal animals.
    4. Rinse 3 times in TBS for 10 min each to rinse out and neutralize the citrate and continue staining.

4. **Mouse antibodies on mouse tissue**
    1. Add 20 µg/ml monovalent Fab fragments α-mouse IgG (H+L; same host-species than secondary antibody) to the first blocking step (*e.g.,* step 3.1.5 or 3.2.1.3).
    2. Rinse 4 times in TBS and once in TBS-T for 10 min each, and proceed with respective protocol.

5. **Cresyl violet counterstaining**
    1. Preheat cresyl violet solution to 60 °C (in glass jar). Incubate slides in the hot solution for 3 min.
    2. Rinse in aq. dest. and dehydrate 2 times for 1 min each in 70%, 96% and 100% isopropanol.
    3. Clear for 5 - 6 min in xylene or a xylene substitute and coverslip with a compatible permanent mounting medium.

4. Data Analysis

1. Count peroxidase stained newborn cells in every 6th section along the entire rostrocaudal extent of the dentate gyrus. Use a light microscope at 400X magnification.
2. Multiply the resulting cell numbers with the intersection interval to obtain an estimate of total numbers of newborn cells.
3. Image the fluorescence labeled sections with a confocal laser microscope equipped with appropriate lasers and filter systems. Take image stacks at random positions along the entire extent of the dentate gyrus at 400X magnification and analyze at least 50 randomly selected cells of interest per hemisphere for co-labeling with other markers.
4. Multiply the resulting percentages of co-labeled cells (%/100) with the total numbers of newborn cells to calculate absolute numbers of specific newborn cell populations.

Representative Results

We applied the methods described above to quantify and characterize newborn cells in the postnatal and adult hippocampus. Therefore, we used wildtype and neurogenesis-deficient cyclin D2 knock out (*Ccnd2*KO) mice housed under conditions known to affect the rate of neurogenesis (*i.e.,* enriched environment, EE)[13,14]. Immunohistochemical DAB staining against either Ki67, BrdU, CldU or IdU consistently revealed differences in newborn cell numbers between wildtype and *Ccnd2*KO mice (**Figure 3**)[15]. Furthermore, with consecutive equimolar injections of CldU and IdU we were able to discriminate cell populations born during specific periods of EE (**Figure 3C,D**). Adopting section 3.2.4 we could successfully detect equal signal intensities and excellent overlap of CldU and IdU after their simultaneous delivery (**Figure 4**). BrdU labeled newborn cells could be phenotyped with a standard immunofluorescence method by either simultaneously applying BrdU and NeuN, (**Figure 5**) or BrdU, GFAP, nestin-GFP and DCX antibodies (**Figure 6B**). This technique allowed successful identification type 1, 2a, 2b and 3 progenitor cells within a single specimen (**Figure 6B,D**). Co-labeling of Ki67 with progenitor markers required the sequential application of antibodies as primary antibodies against Ki67 and GFAP were raised in rabbit (applies to section 3.2.2), moreover one of the primary antibodies (goat α-GFP) was produced in the same host as one of the secondary antibodies (AMCA conj. goat α-rabbit). A combination of sections 3.2.2 and 3.2.3 perfectly worked if sections were incubated first in Ki67 together with nestin-GFP antibody, and secondly in GFAP antibody but not vice versa (**Figure 6C**). **Figure 2B** summarizes the approved application schemes.

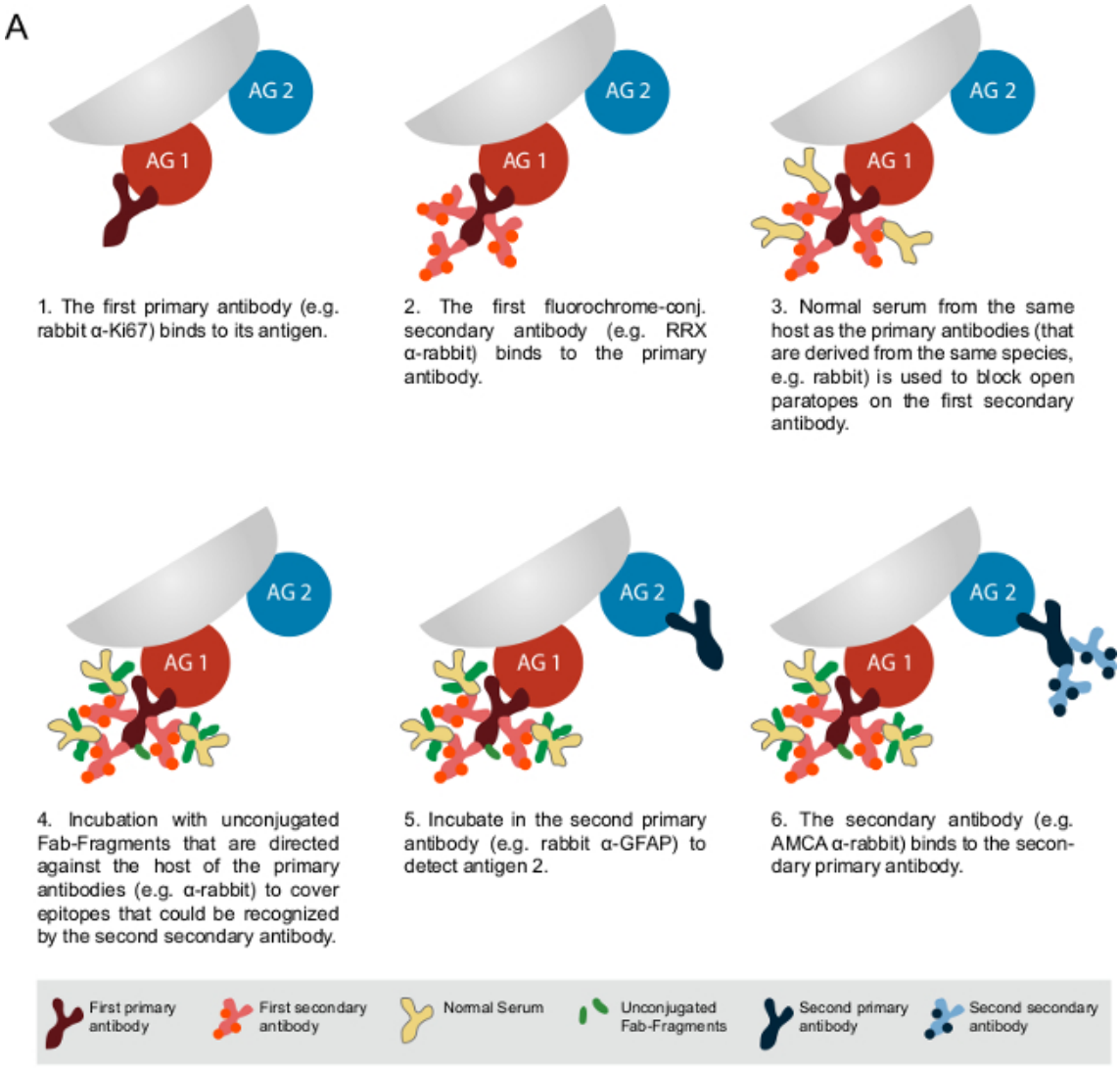

Figure 2. Sequential multiple immunofluorescence with primary antibodies derived from the same host species. (A) Schematic illustration. **(B)** Successful combinations of antibodies and their chronological order in immunofluorescence. Please click here to view a larger version of this figure.

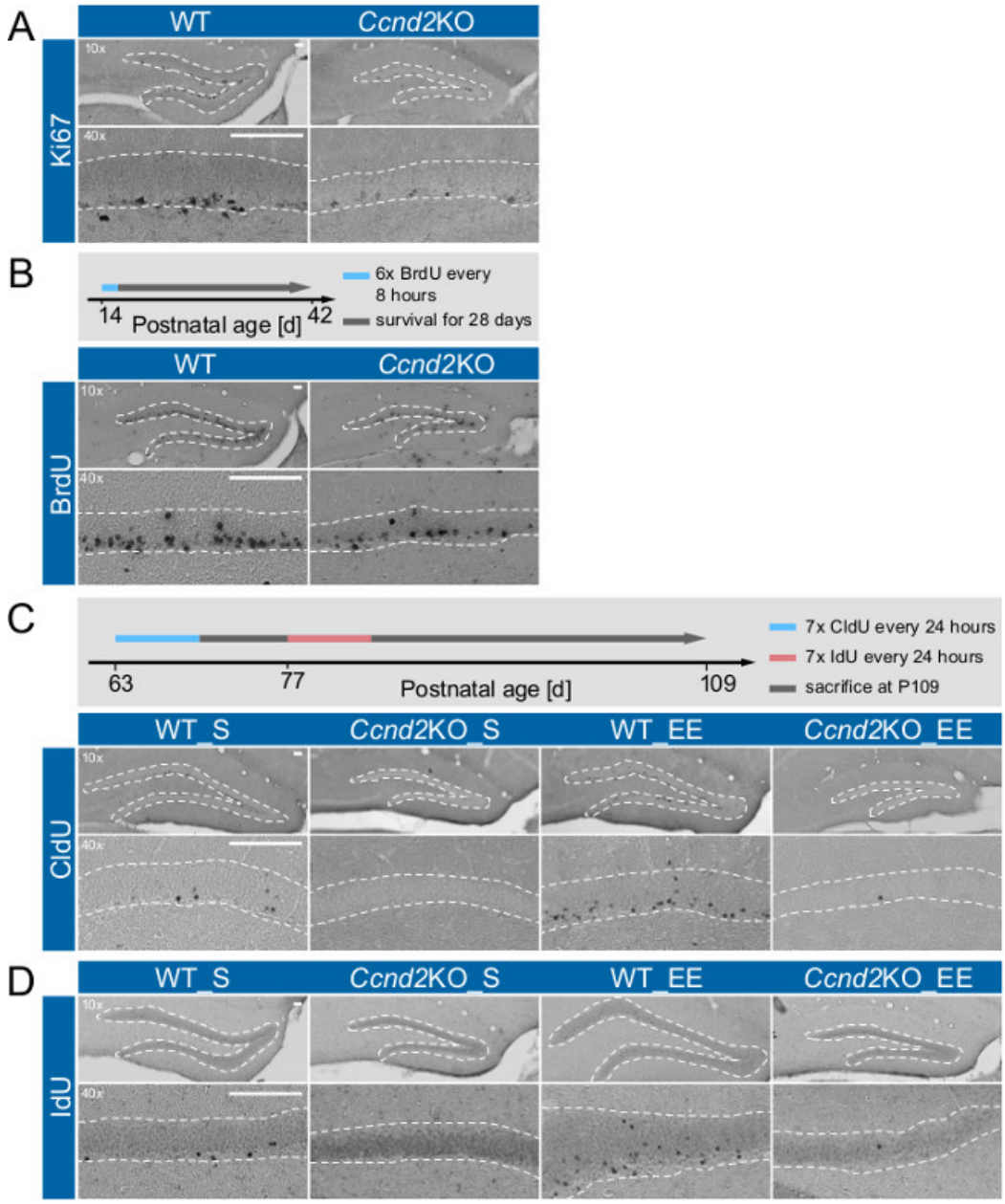

Figure 3. Light microscopy images of ABC-immunohistochemistry for quantification of newborn cells in the dentate gyrus. Depicted are comparisons of different proliferation markers in WT and neurogenesis-deficient *Ccnd2*KO mice at 100X and 400X magnification. **(A)** Ki67-DAB staining shows clusters of proliferating cells in the subgranular zone of the dentate gyrus (postnatal day 35). The number of proliferating cells is diminished in *Ccnd2*KO mice compared to WT mice. **(B)** Newborn cells at 28 days after BrdU-administration (injection scheme above images) confirming the reduced proliferation rate in *Ccnd2*KO mice. **(C)** CldU-administration during the 6th week of EE (injection scheme above image) revealed an increase in the number of newborn cells in the dentate gyrus of WT but not *Ccnd2*KO mice. **(D)** IdU-administration during the 8th week of EE showed similar results. Scale bar represents 50 µm. Please click here to view a larger version of this figure.

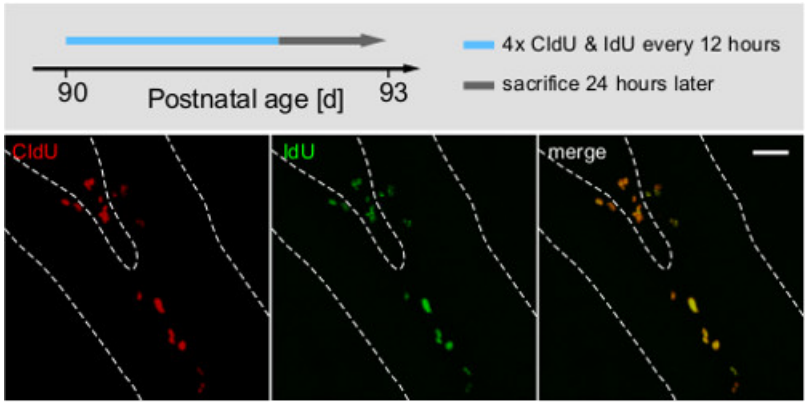

Figure 4. Immunofluorescence co-staining of CldU and IdU after simultaneous delivery of equimolar concentrations. Signal amplification of CldU labeled cells resulted in an excellent overlap of CldU and IdU. Scale bar represents 20 µm. Please click here to view a larger version of this figure.

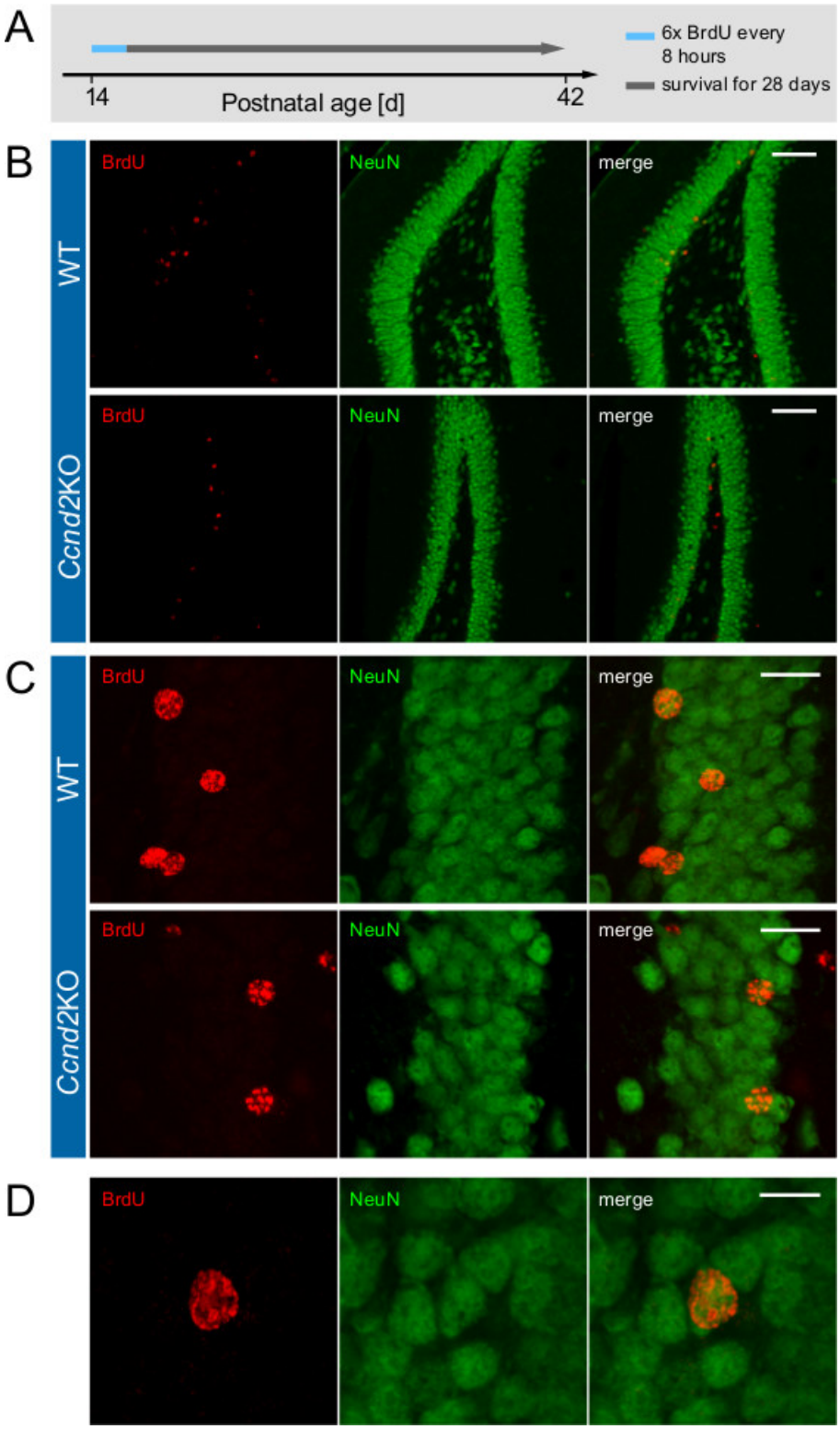

Figure 5. Immunofluorescence co-labeling of NeuN and BrdU. (A) BrdU was administered 6 times every 8 hr for 2 consecutive days starting at postnatal day 14. Animals were sacrificed 28 days later. **(B)** Immunofluorescence co-labeling of proliferating cells with the mature neuronal marker NeuN shows a reduced number of proliferating cells in *Ccnd2*KO compared to WT animals. Scale bar represents 50 µm. **(C)** Enlarged view of (B) showing co-labeled cells in both genotypes. Scale bar represents 20 µm. **(D)** High magnification example of a BrdU/NeuN co-labeled cell in the dentate gyrus. Scale bar represents 10 µm. Please click here to view a larger version of this figure.

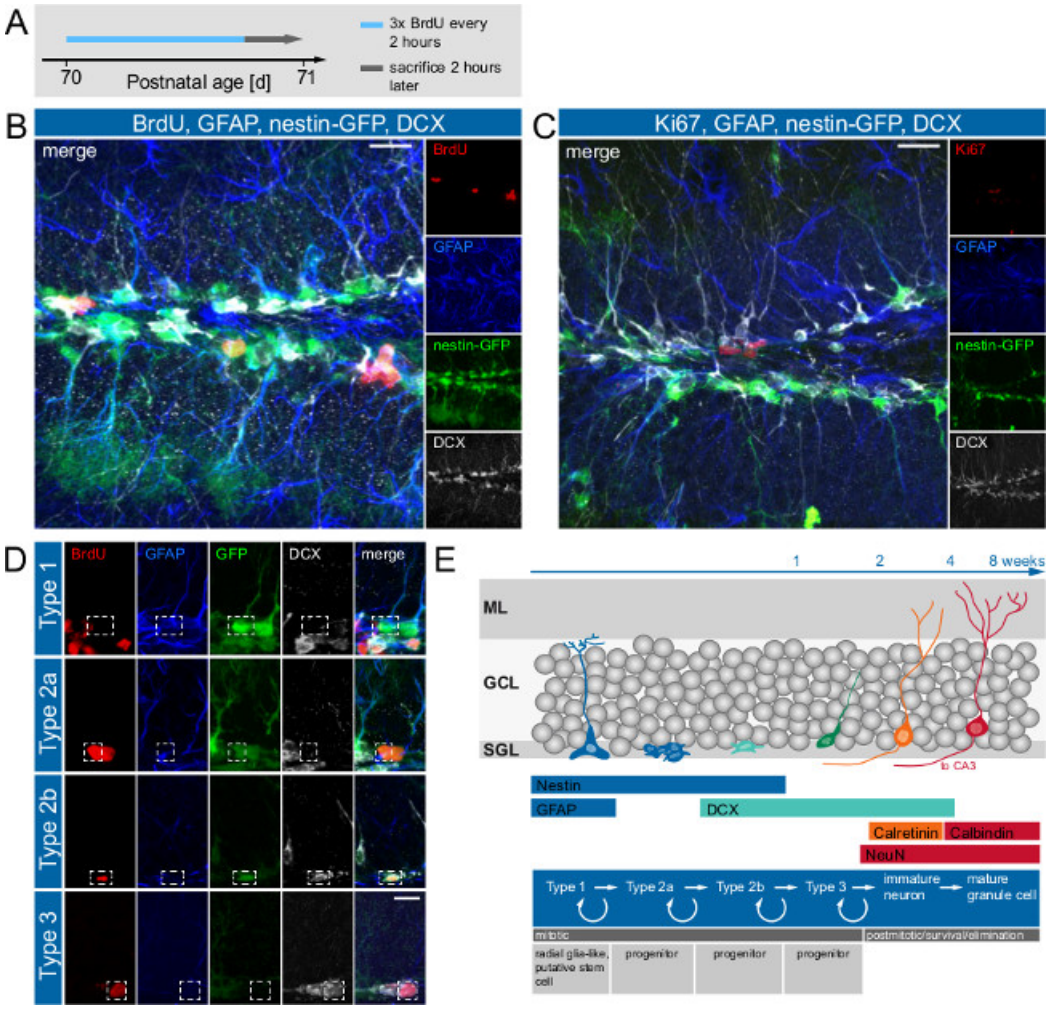

Figure 6. Quadruple immunofluorescence co-labeling of different proliferation markers with GFAP, nestin-GFP and DCX for phenotyping newborn granule cells in the dentate gyrus of nestin-GFP mice. (A) BrdU was administered 3 times every 2 hr at postnatal day 70. Animals were sacrificed 2 hr later. **(B)** Representative image of a co-staining for BrdU, GFAP, nestin-GFP and DCX. **(C)** Representative image of quadruple immunofluorescence for Ki67, GFAP, nestin-GFP and DCX. **(D)** Classification of progenitor cell types according to their immunofluorescence labeling. Based on the co-expression of different markers and the morphological features of the labeled cells four distinct progenitor cell subtypes can be identified: type 1 ($GFAP^+$, $nestin^+$, DCX^-), type 2a ($GFAP^-$, $nestin^+$, DCX^-), type 2b ($GFAP^-$, $nestin^+$, DCX^+) and type 3 ($GFAP^-$, $nestin^-$, DCX^+). Scale bar represents 20 µm. **(E)** Schematic overview of the different stages of adult hippocampal neurogenesis. It illustrates the 4 progenitor cell types, immature neurons and mature granule cells, their characteristic expression of markers and their proliferative capacity. ML – molecular cell layer, GCL – granular cell layer, SGL – subgranular cell layer. Please click here to view a larger version of this figure.

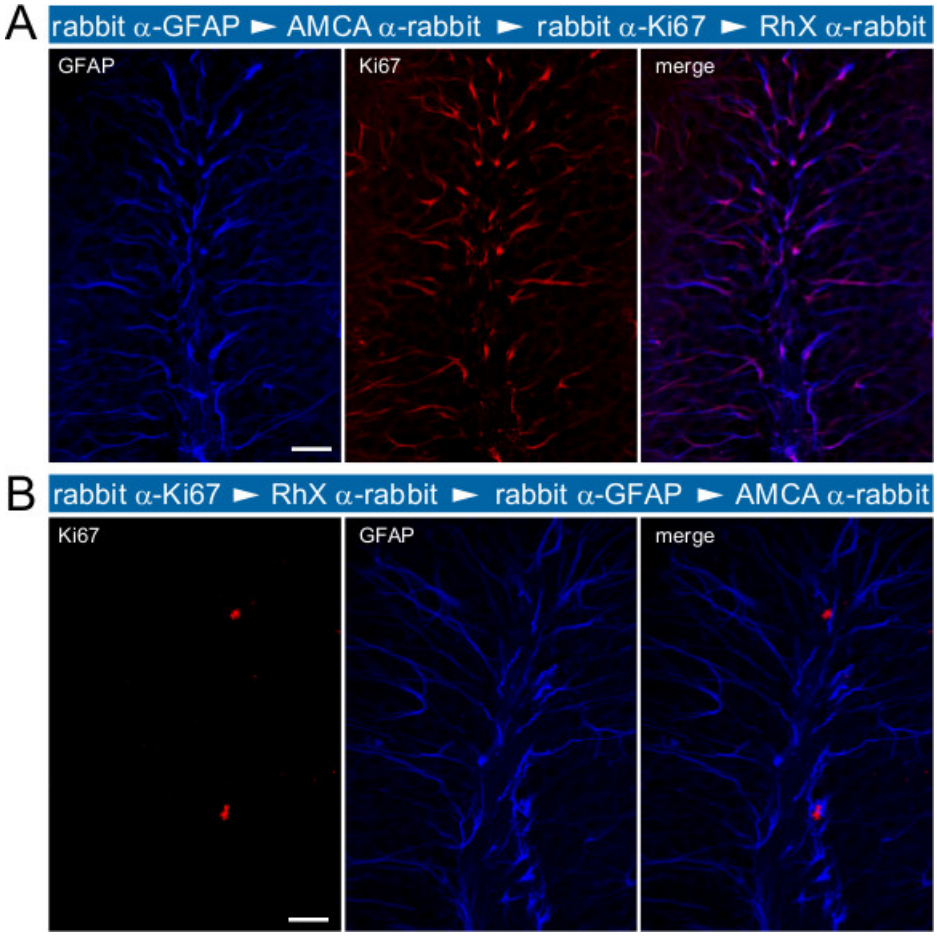

Figure 7. Example of successful and non-successful sequential stainings with two primary antibodies raised in the same species, depending on the sequence of antibody incubation. The confocal micrographs show the outcome of a sequential co-staining against Ki67 and GFAP with primary antibodies both derived from rabbit. In **(A)** immunohistochemistry was first completed for GFAP followed by staining against Ki67 which led to a pronounced overlap of the two signals. In particular the Ki67 signal was atypical and resembled the signal of the GFAP staining. **(B)** shows the results from the reverse staining sequence with the Ki67 staining completed first and subsequent staining against GFAP. Here, the signals for both antigens resembled the expected expression pattern: The Ki67 signal was restricted to nuclei within the subgranular zone while the GFAP signal was found cytoplasmatically, mainly in the cellular processes arising from the subgranular zone. Scale bar represents 20 µm. RhX – Rhodamine X. Please click here to view a larger version of this figure.

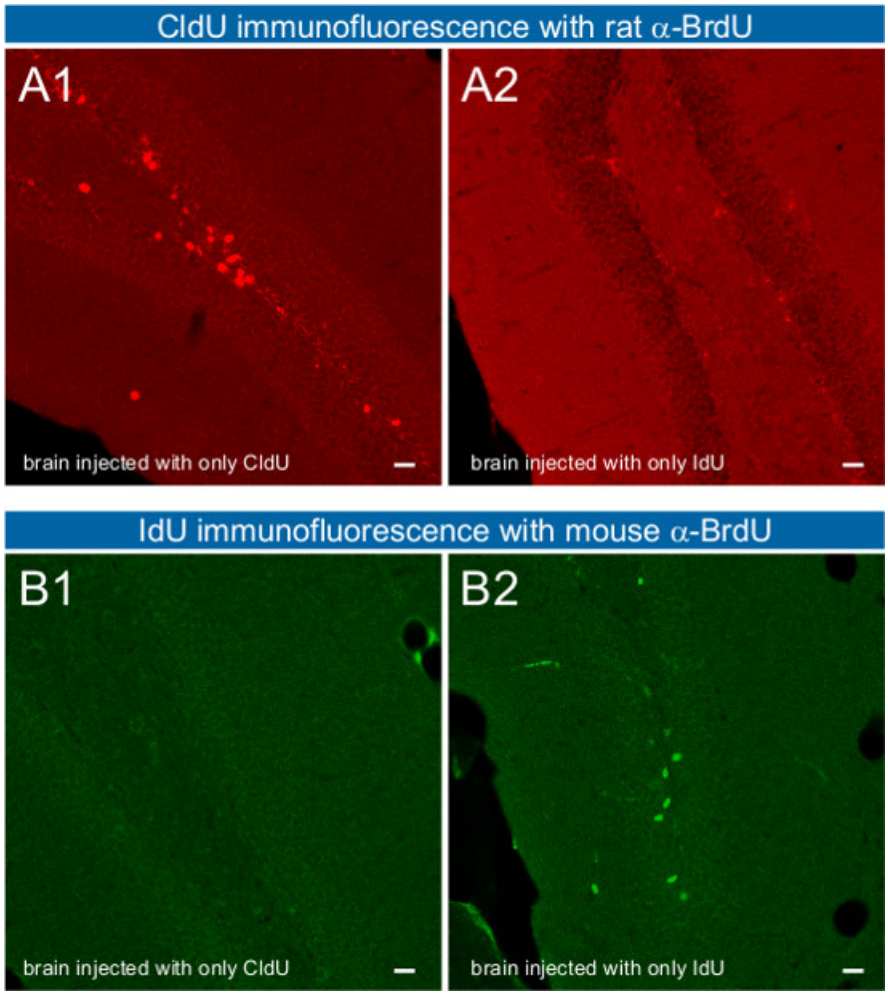

Figure 8. Specificity of the α-BrdU antibodies used to detect CldU and IdU. Mice were injected either with CldU or with IdU (non-simultaneous application). In **(A)** brain sections of CldU (A1) or IdU (A2) injected mice were immunostained using the purified rat α-BrdU antibody which should specifically cross-react to CldU, but not to IdU. **(B)** shows the results from staining brains from CldU (B1) or IdU (B2) injected mice with the mouse α-BrdU antibody that is expected to cross-react with IdU, but not CldU. Scale bar represents 20 µm. Please click here to view a larger version of this figure.

Discussion

Quantification and identification of subpopulations of newborn cells is a central issue in adult neurogenesis research. Combining proliferation markers and antibodies against proteins expressed during specific stages of adult neurogenesis allows immunohistochemical detection of these subpopulations. Some of the antibodies or antibody combinations require specific staining conditions.

Labeling of dividing cells with synthetic thymidine analogs is still the gold standard for studying adult hippocampal neurogenesis. It is crucial to consider the appropriate injection protocol prior to start of the experiment. We usually administer 50 mg/kg (body weight, i.p.) BrdU, but concentrations may differ depending on experimental requirements (up to 300 mg/kg for a bolus injection)[8,16]. If IdU and CldU have to be injected sequentially for temporal discrimination of cell populations it is mandatory to inject equimolar concentrations (*e.g.,* 42.5 mg/kg CldU and 57.5 mg/kg IdU). Note that thymidine analogs may be toxic to dividing cells and may also label damaged cells undergoing DNA synthesis[16]. Another disadvantage is that DNA denaturation is required for immunohistochemical detection of thymidine analogs which might interfere with the antigenicity of antibodies or other DNA labeling techniques (*e.g.,* DAPI, Hoechst 33258). Immunohistochemical detection of endogenous proliferation markers like Ki67 may be an adequate alternative[10,11]. However, this only allows a snap shot of proliferative activity at the time of perfusion, retrospective birth dating and fate analysis are impossible.

For immunostaining, sections are generally processed free floating, usually in 6-well plates equipped with a carrier plate and mesh inserts which simplifies their transfer from one solution to another. As an exception, blocking, antibody incubations and ABC reaction are done in 12- or 24-well plates without mesh inserts to economize expensive solutions (0.5 to 1 ml per well is sufficient, depending on the number of slices that have to be stained). During these steps, transfer sections with the help of a fine brush that needs to be rinsed when changing solutions. Prevent sections from drying-out and perform incubations that take >1 hr in a humified chamber. All incubations have to be done with continuous agitation (max 150 rpm). Always test and titrate each new antibody lot. Longer antibody incubation times (up to 2 days at 4 °C) may improve staining. In case only mouse antibodies are available against antigens you want to visualize in mouse tissue it is advisable to block endogenous immunoglobulins with highly concentrated monovalent Fab fragments α-mouse (section 3.4). Whenever using this technique extend the following

rinsing steps. Otherwise, unbound Fab fragments might bind to your primary mouse antibody. HCl pretreatment and borate neutralization are required only for detection of thymidine analogs. The 12 min peroxidase reaction result in an optimal signal-to-noise ratio with the conditions and antibodies applied in our protocol. As with any enzymatic reaction, the rate of the peroxidase reaction depends on many factors (*e.g.,* temperature, pH, concentration of enzyme and substrate). Consequently, reaction times need to be optimized for any new antibody set. To visualize anatomical structures in DAB stains with very weak background (*e.g.,* CldU), slices can be counterstained. The cresyl violet method described in section 3.5 results in a very faint counterstain that does not compromise the specific DAB signal.

For multiple immunofluorescence, use primary antibodies that are raised in different species or of different isotypes and follow section 3.2.1. Otherwise, perform a sequential multiple immunolabeling which has been optimized to detect multiple primary antibodies from the same host species (*i.e.,* mouse or rabbit; section 3.2.2). The basic principle of this method has been invented by Ferguson and colleagues who successfully stained different muscle markers with two mouse primary antibodies[17]. The Fab fragments used for blocking in step 3.2.2.5 should be derived from the same host species as the secondary antibodies. The concentrations and incubation times described in section 3.2.2 are optimized for the specific antibodies described herein and have to be adjusted for any new antibody combination. Be aware that the sequence of primary antibody incubation might strongly influence the outcome (as indicated in **Figure 7A**). Wherever applicable, the antigens detected by the primary antibodies that are derived from the same species should show a different expression pattern which simplifies the detection of insufficient blocking. To eliminate any possibility of antibody cross-reaction or false-positives it is essential to include appropriate controls (*i.e.,* only-secondary-antibody controls; complete immunohistochemistry for first antigen followed by an incubation with second primary antibody or second secondary antibody alone)[18]. **Figure 2B** shows antibody combinations that worked well in our hands. For multiple immunofluorescence it is also advisable to use secondary antibodies that are raised in the same species (*e.g.,* donkey) and have minimal cross-reactivity to your tissue and to serum proteins from other species (*i.e.,* pre-adsorbed). Otherwise section 3.2.3 may help to prevent any unexpected interspecies cross-reactivity. Select fluorochromes with minimal spectral overlap appropriate to your detection system (*e.g.,* AMCA, Alexa Fluor 488, Rhodamine Red, Alexa Fluor 647). If a nuclear counterstain is required, add DAPI or Hoechst 33342 (10 µg/ml) to the secondary antibody cocktail (then, no near-UV secondary antibody can be used). These nuclear counterstains do not work if tissue has been pre-treated with HCl. Similarly, HCl treatment may compromise the detection of some antigens and therefore its influence on individual antibody performance needs to be tested. Once the fluorochrome-conjugated secondary antibodies have been applied protect sections from light.

CldU and IdU can be visualized with the help of two different BrdU antibodies that cross-react either with CldU (rat α-BrdU) or IdU (mouse α-BrdU)[9,19]. If both thymidine analogs have been injected into one animal it is absolutely essential to use the purified rat α-BrdU antibody to detect CldU because the non-purified antibody cross-reacts to IdU. As described by Vega *et al.*[9] the CldU signal needs to be amplified for immunofluorescent co-labeling to achieve equivalent detection of both thymidine analogs. Therefore, instead of a fluorochrome-conjugated secondary antibody (α-rat) a fluorochrome conjugated streptavidin is added following incubation in a biotinylated secondary antibody (α-rat; section 3.2.4). To exclude any undesired antibody cross-reactions include control sections from mice that received either IdU or CldU (separately; see **Figure 8**). To verify equivalent detection of IdU and CldU use sections from animals that were simultaneously injected with equimolar amounts of CldU and IdU (see **Figure 4**).

To study type 1 and type 2 progenitor cells we prefer to use nestin-GFP transgenic mice[12] as nestin antibodies frequently did not provide satisfactory results. Nestin-GFP mice have the advantage of reliably visualizing nestin-positive progenitor cells and their morphological features at a particular developmental stage. The GFP-signal needs to be amplified through indirect immunofluorescence with an α-GFP antibody. The secondary antibody should be coupled to a fluorochrome with spectral properties similar to GFP. If required, nestin-GFP mice can be crossbred to other mutant mouse lines to investigate the involvement of particular genes in neurogenesis. By now, improved nestin antibodies are supplied which label cell bodies as well as processes and can be used as an alternative to the transgenic approach (see material list).

It is critical to comply with the 24 hr fixation to prevent overfixation. The chemical reaction partners of formaldehyde in tissue are primarily proteins and formaldehyde generates reactive hydroxymethyl groups on their side chains leading to cross-linking of adjacent amino acids (by the formation of methylene bridges). This may cause masking of antigen-epitopes and loss of immunoreactivity (see comments to specific antibodies in the **Materials List**). To recover lost immunoreactivity, several epitope retrieval methods exist that break formaldehyde-induced protein cross-linkages through exposure to heat[20]. In this protocol, the citrate buffer method works superior to others for detection of neurogenesis in the postnatal and adult hippocampus (section 3.3). Note that slices of very young animals (< P21) may become very fragile or distorted and need to be handled with special care. Furthermore, epitope retrieval may be detrimental for some epitopes and therefore individual antibodies should be tested whether they are suitable for multiple staining that requires this pre-treatment.

In summary, this protocol is a collection of basic and elaborated techniques that facilitate the analysis of adult hippocampal neurogenesis. It particularly allows an unequivocal identification of all hippocampal progenitor subpopulations together with a proliferation or birth-dating marker from a single section. These techniques can be adapted to any combination of antibodies or to other species to study the development and regulation of hippocampal progenitor cells in response to specific stimuli and their involvement in particular brain functions.

Disclosures

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