Protocol

Proliferation markers in the adult rodent brain: Bromodeoxyuridine and proliferating cell nuclear antigen

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Abstract

The rostral migratory stream is one of the few regions of the adult mammalian central nervous system in which cellular migration and proliferation have been described. Most rostral migratory stream cells divide rapidly and hence different proliferation markers have been employed to identify them. Nitrogen base substitutes, such as tritiated thymidine or 5-bromo-2'-deoxyuridine (BrdU), together with endogenous molecules, such as Proliferating Cell Nuclear Antigen (PCNA), are the cell cycle markers most widely employed. Protocols for BrdU and PCNA localization are both plentiful and diverse, but to date no optimized protocol for obtaining trustworthy double staining of both markers has been described. In this work, we propose optimized protocols for achieving both single staining and the joint detection of BrdU and PCNA in the rodent brain using double-immunofluorescence procedures. The double labeling described allows the discrimination of different cell cycle stages in migratory cells from the mouse brain.

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1. Type of research

The rostral migratory stream (RMS) is a region of the mammalian brain where neuroblasts migrate tangentially towards the olfactory bulb. Once there, these progenitor cells migrate radially and finally differentiate into interneurones [3]. The RMS is located in the rostral extension of the subependymal layer of the lateral ventricle and is mainly formed by astrocytes and neuroblasts [16,20,34,44]. Only neuroblasts divide in this region and there they migrate forward, forming chains. In contrast, the function of astrocytes is both to wrap and to guide such migrating cells [34]. Owing to the high rate of cell division in this region, proliferation markers are frequently employed to study and analyze RMS cells. Proliferation markers such as tritiated thymidine ([^3]H-T) were first used by Taylor to analyze chromosomal replication [52–54] and later employed by different authors to demonstrate the existence of neural proliferation in the mature central nervous system of mammals [1,2].

Two groups of cell proliferation markers can be distinguished: analogs of nitrogen bases and endogenous proliferation markers (cell cycle-related molecules only expressed by proliferating cells). Currently, the nitrogen base analog most widely used for labeling proliferating cells is 5-bromo-2'-deoxyuridine (BrdU) [17–19,21,23,43]. It is a non-radioactive thymidine analog that acts like[^3]H-T but that can be detected using immunohistochemical techniques [4–7,17,48]. BrdU has been used since the 1970s as a tool for measuring DNA synthesis in isolated...
chromosomes, cells and tissues [17]. Polyclonal antibodies have been raised to detect BrdU incorporated into DNA strains. However, these initial antisera were heterogeneous and contained other immunoglobulins that also bound to unmodified DNA [25]. This problem was solved by the raising of mouse monoclonal antibodies that were highly specific for BrdU [21,23,56]. Currently, well-characterized BrdU antibodies have low cross-reactivity with double-stranded DNA, and for the proper detection of DNA, this must first be denatured. HCl, NaOH or certain enzymes are the treatments most commonly employed to obtain good results [25]. Nonetheless, protocols aimed at both the denaturing of DNA and the proper detection of BrdU are manifold and there is no consensual step-by-step procedure. Other proliferation markers are endogenous to the cells themselves and their expression fluctuates along the different cell cycle phases [9]. The most widely employed endogenous proliferation marker is Proliferating Cell Nuclear Antigen (PCNA), an auxiliary protein of DNA polymerase $\delta$ [10,46]. The half-life of PCNA is about 20 h, and its expression begins to increase during the late G1 and early S-phases and declines throughout G2 and mitosis [27,32,33]. PCNA has been described to be present in some proliferating cells even at G0, although its expression is very reduced [9]. No special treatment is required to detect PCNA in tissue, although different types of nuclear labeling have been observed in vitro when cells are fixed either with methanol or with formaldehyde. PCNA has been localized associated with both DNA replication foci and free in the nucleus when tissues are fixed with formaldehyde [9]. However, methanol fixation prevents the detection of PCNA unbound to DNA in cell cultures [9].

The aim of the present work is to assess an optimal protocol for the simultaneous detection of BrdU and PCNA in the adult mouse brain, testing different fixatives and tissue treatments. The combination of both markers permits study of the proliferative, migratory and survival characteristics of neuronal progenitor cells such as those of the RMS.

The approach used in this work should be useful for the following types of studies:

I. Detection of proliferating cells and analysis of both the proliferation frequency (using PCNA immunohistochemistry) and the survival of progenitor cells that arrive at the olfactory bulb from the RMS (using BrdU immunohistochemistry).

II. Studies of active replication regions of DNA in fixed tissues and localization of nuclear proteins, such as PCNA, related to these regions.

III. Detection of cell cycle phases and estimation of cell cycle length using several BrdU injection protocols.

IV. Analysis of PCNA distribution in the different nuclear compartments.

2. Time required

Two BrdU administration protocols were employed in two different experimental groups: single intraperitoneal injection 30 min before animal sacrifice and three i.p. injections 26, 14 and 2 h before sacrifice.

The time required to perform the entire procedure (excluding the BrdU injections) is 4 days.

3. Materials

3.1. Animals

80-day-old male CD-1 mice (Mus musculus, L. 1758) were used. The animals were always housed in conditions of constant temperature and humidity under a 12/12 h artificial photoperiod and were fed ad libitum with water and composite fodder (Rodent toxicology diet, B&K Universal G.J., S.L. Molins de Rei, Barcelona, Spain). The animals were kept, handled and sacrificed in accordance with current European (directive 86/609/EEC) and Spanish legislation (BOE 67/8509-12, 1988).

3.2. Special equipment

- Leica Frigomobil Jung SM 2000, Nussloch, Germany.
- Perfusion peristaltic pump (Miniplus 3 M312, Gilson; Villiers-le-Bel, France).
- Leica TCS SP2 spectral confocal microscope (Leica Mannheim, Germany).

3.3. Chemicals and reagents

Chemicals:

- 5-Bromo-2′-deoxyuridine (#B-5002 Sigma Chemical Co., St. Louis, USA)
- 5-Fluorodeoxyuridine (#F-0503 Sigma)
- Acetic acid (#58 Merck, Darmstadt, Germany)
- Chloral hydrate (#22 682.265 Prolabo, Fontenay-St.-Bois, France)
- D-(-)-Sucrose (#141.621 Panreac Montplet and Esteban S.A., Barcelona, Spain)
- 99% glycerol (#211339 Panreac)
- 98% glycine (#G6201 Sigma)
- 35% hydrochloric acid (#211019 Panreac)
- 33% hydrogen peroxide (#096010 Probus S.A., Barcelona, Spain)
- N-propyl gallate (#P-3130 Sigma)
- Paraformaldehyde (#222010 Probus)
- Picric acid (#014220 Probus)
- Polyethylene glycol 400 (#162436.1611 Panreac)
- Sodium borohydride (#S-9125 Sigma)
- Sodium chloride (#27.800.291 Prolabo)
- Sodium hydroxide (#28.245.298 Prolabo)
- Thymersal (#T-8784 Sigma)
- Triton® X-100 (#28.817.295 Probus)

Antibodies and serum:
- Cy2-conjugated anti-rat-IgG raised in goats (#112-225-003, Jackson Laboratories, West Grove, PA, USA)
- Cy3-conjugated anti-mouse-IgG raised in goats (#115-165-003, Jackson Laboratories)
- Normal goat serum (#S-1000, Vector Laboratories, Burlingame, CA, USA)
- Mouse anti-PCNA monoclonal antibody (#(PC10)sc-56, Santa Cruz Biotechnology Inc., Santa Cruz, California, USA)
- Rat anti-BrdU monoclonal antibody (#MAS250c Accurate Chemical and Scientific Corporation, New York, USA)

4. Detailed procedure

4.1. BrdU administration

Each animal was injected intraperitoneally with 30 μg/g b.w. of BrdU and 3 μg/g b.w. of 5-fluorodeoxiuridine (FdU) dissolved in 0.1 M phosphate-buffered saline, pH 7.4 (PBS). This solution was administered either 30 min before animal sacrifice or 26, 14 and 2 h before sacrifice. The single injection protocol was employed to stain and distinguish active replication sites in S-phase cells [42]. The three-injection protocol was used to label the maximum number of proliferating cells with BrdU and that combined with PCNA-immunostaining was employed to investigate whether PCNA was present exclusively in proliferating cells.

4.2. Fixation

After previous probes, we concluded that the optimum fixative for the detection of BrdU and PCNA is a variant of Bouin’s fixative [8], called Bouin-4%, made up of 4% (w/v) depolymerized paraformaldehyde, 1% (w/v) picric acid and 5% (v/v) acetic acid (the original formula contains 8% formaldehyde).

I. Animals were deeply anaesthetized with 10 μl/g b.w. of 5% (w/v) chloral hydrate in 0.9% NaCl and then perfused intracardially with 0.9% NaCl, followed by 5 ml/g b.w. of Bouin-4% fixative.
II. After perfusion, brains were dissected out and post-fixed with the same fixative for 1 h.
III. The brains were then rinsed for 2 h with 0.1 M phosphate buffer, pH 7.4 (PB).

4.3. Preservation and tissue sectioning

I. Brain blocks were cryoprotected by immersing them in 30% (w/v) sucrose in PB.
II. When they had sunk, the blocks were stored frozen at −80 °C until sectioning.
III. 40 μm-thick sections were obtained with a freezing-sliding microtome.
IV. The sections were immunostained or stored at −20 °C in a freezing mixture made up of 30% glycerol and 30% polyethylene glycol in PB.

4.4. Immunohistochemistry

I. Freeze-stored sections were warmed up to room temperature and then thoroughly rinsed 5 × 10 min with PB.
II. Sections were incubated with 0.13 M sodium borohydride for 20 min to eliminate aldehyde autofluorescence.
III. Tissue slices were washed 3 × 10 min in PBS.
IV. Sections were incubated in 2 N HCl at 37 °C for 1 h to denature DNA, allowing the antibody to bind to the incorporated BrdU.
V. The sections were then rinsed 3 × 10 min in 0.1 M borate buffer (pH 8.5) to neutralize HCl acidity.
VI. The tissue was then washed 3 × 10 min in PBS to stabilize the pH.
VII. The tissue was incubated at 4 °C with the primary antiserum for 48 h. The incubation medium contained: 1:5000 rat anti-BrdU, 1:3000 mouse anti-PCNA, 0.2% (w/v) Triton X-100 and 5% (v/v) normal goat serum in PBS.
VIII. Tissue slices were rinsed 3 × 10 min in PBS.
IX. The tissue was then incubated at room temperature in the dark, for 2 h in the secondary antiserum. The incubation medium was made up of 1:200 Cy2 goat anti-rat IgG, 1:200 Cy3 goat anti-mouse IgG and 0.2% (w/v) Triton X-100 in PBS.
X. The tissue was rinsed in the dark 3 × 10 min in PBS.
XI. Slices were finally mounted with coverslips using a freshly prepared anti-fading medium containing 0.42% (w/v) glycine, 0.021% (w/v) sodium hydroxide, 0.51% (w/v) sodium chloride, 5% (w/v) N-propyl-gallate, 70% (v/v) glycerol and 0.002% (w/v) Thymersal in distilled water.

5. Results

5.1. BrdU staining

5.1.1. Single injection of BrdU

This BrdU administration protocol allowed the detection of cells in S-phase (Fig. 1). BrdU-labeled cells of...
the RMS showed three staining patterns similar to the three S-phase stages previously described in vitro [35,41]:

a) Early S-phase cells (Fig. 1A, solid arrow). These cells were characterized by a large number of punctuate spots throughout the nuclei [35,41,42].

b) Mid S-phase cells (Fig. 1A, solid arrowhead). These cells were identified by their perinuclear, perinucleolar or intranucleolar rims of numerous small and discrete granules [35,41,42].

c) Late S-phase cells (Fig. 1A, open arrow). These cells showed large granular spots (“patches”) in their nuclei [35,41,42].

5.1.2. Three injections of BrdU

The number of cells that incorporated BrdU into their DNA increased considerably using the three-injection protocol, as expected (Fig. 2). Using this injection protocol,
BrdU staining patterns from different S-phase stages were seen in the same nucleus (Fig. 2A, open arrowhead). Since RMS proliferating cells have a cell cycle length of approximately 17.3 h [49], this pattern presumably appears in cells that incorporate BrdU twice, in consecutive cell cycles.

5.2. PCNA staining

PCNA staining was similar using the one- or three-BrdU injection protocols (Figs. 1B and 2B). The presence of both DNA-associated PCNA and free PCNA (Figs. 1B and 2B) rendered PCNA staining less useful to identify the three S-phase stages described above with BrdU labeling. Nevertheless, the patchy pattern of the late S-phase stage was readily distinguished.

5.3. Double BrdU/PCNA staining

5.3.1. Single injection of BrdU

Double-immunostaining analysis revealed that BrdU colocalized tightly with PCNA in all nuclei, no cells labeled exclusively with BrdU being observed (Fig. 1C). Using the single-BrdU injection protocol, BrdU labeled S-phase cells, staining active DNA replication regions, so PCNA was present associated with replication sites in all S-phase nuclei. Quantitative analyses revealed that only 36% of the total PCNA positive nuclei were double-labeled for both PCNA and BrdU (Fig. 3). The existence of cells single-labeled for PCNA confirmed that PCNA was not only present in S-phase cells.

5.3.2. Three injections of BrdU

When double-immunostaining for BrdU and PCNA was studied using the three-BrdU injection protocol, the PCNA distribution pattern was not always coincident with that of BrdU (Fig. 2C). Thus, some nuclei displayed a late S-phase phenotype of PCNA distribution combined with a BrdU pattern presumably corresponding to “early” or “mid S-phase” stages (Fig. 2C). This type of labeling corresponded to proliferating cells that had progressed through the cell cycle after they had incorporated BrdU.

Using this triple BrdU injection protocol, the number of cells double-stained for PCNA and BrdU was found to be higher (88%; Figs. 2C and 3), confirming that these PCNA-positive proliferating cells had duplicated their DNA. However, using this injection protocol, cells single-stained for BrdU were detected (Fig. 2, solid arrowhead). These cells were in G0 or early/mid G1, because PCNA is very reduced and undetectable in nuclei during these cell-cycle phases [9]. Progenitor cells of the RMS undergo successive rounds of division and differentiation as they migrate through the RMS before becoming permanently postmitotic [15]. Accordingly, cells of the RMS single-labeled for BrdU incorporated it during S-phase and then progressed through the cell cycle to a semi-differentiated state, down-regulating PCNA expression.

6. Discussion

6.1. Overall assessment of the protocol

PCNA antibody clone PC10 has been described to stain proliferating and non-proliferating cells [29], although it has been widely employed in studies on cell proliferation [13,24,27,40,45]. Our results point to the importance of the fixation procedure for obtaining adequate detection of PCNA in tissues using this antibody. Several fixatives were tested, such as 4% paraformaldehyde with or without 0.1% or 0.2% (v/v) glutaraldehyde; Somogyi’s solution [51] without glutaraldehyde; Bouin’s solution [8] with different paraformaldehyde concentrations (1, 4, 8 and 10% w/v); Carnoy’s solution [12] and ethanol–formaldehyde [14]. Carnoy’s solution and ethanol–formaldehyde have been used previously to detect nuclear components [14,28] but they did not preserve the tissue adequately. On using Somogyi’s or paraformaldehyde, RMS cells were intensely labeled but cells surrounding RMS, most of them mature neurons and glia, were also observable. Bouin-4% fixative specifically allowed the detection of PCNA associated with cell proliferation and it was also useful for labeling BrdU with high resolution in the mouse RMS. This fixative also permits the detection of both the fraction of PCNA that was associated and that unassociated with replication sites, allowing the staining of both pure S-phase cells and those entering or leaving this phase. Thus, using this PCNA detection protocol, a better estimation of the total number of proliferating cells in a given brain region can be achieved. When the three-BrdU injection protocol was applied, the high rate of double-stained (PCNA/BrdU) cells indicated that PCNA-labeling was only present in proliferating cells (Figs. 2C and 3). The precise mechanism by which Bouin-4% eliminates non-specific staining in non-

![BrdU/PCNA-double labelled cells](image_url)

Fig. 3. Percentage of RMS cells stained for PCNA that are double-labeled for BrdU and PCNA in mice injected with BrdU once or three times before sacrifice. Error bars indicate SEM. When animals were injected three times with BrdU, the number of BrdU/PCNA-double stained cells increased ($P < 0.01$ for significant differences according to Student’s $t$ test).
proliferating cells is unknown, but the properties of each component of Bouin’s solution have been well described. Formaldehyde is known to react with arginine and lysine residues, which are very abundant in histones [26,30]. Furthermore, Bouin’s solution contains a high proportion of picric acid, which is also known to react with histones, forming crystalline picrates with their amino acids [28]. Well preserved histones can maintain an adequate structure of DNA and its associated molecules, thus allowing a good detection of BrdU and PCNA. Finally, acetic acid contributes to a better fixation of nuclear components since it is characterized by an elevated coefficient of diffusibility and penetrates tissues easily [28,38]. Moreover, the addition of acetic acid renders Bouin’s solution much more acidic. The low pH of Bouin-4% fixative could be necessary to obtain a good immunostaining [26,28].

BrdU can be administered to live animals in their drinking water, or by intraperitoneal injections. BrdU doses vary between 25 and 600 μg/g of animal weight [11,22,47]. Exposure of embryos to high doses of BrdU affects body and brain morphology [31]. The use of low BrdU doses is recommended because multiple doses of 50–500 μg/g (often used with adult mammals) may produce several artifacts [47]. Nevertheless, specific research requires the use of higher BrdU doses (up to 300 μg/g) to label most S-phase dividing cells in the adult central nervous system [22]. BrdU has been also employed jointly with FdU [55], an inhibitor of thymidine synthesis [37]. Dividing cells exhibit a greater avidity for BrdU when the availability of thymidine is limited. Thus, lower doses of BrdU can be employed if combined with FdU [19], overcoming the mutagenic effects of BrdU and poor incorporation into dividing DNA [36]. In the proposed protocol, a low dose of BrdU was mixed with FdU to afford the labeling of all S-phase cells. S-phase staining patterns for PCNA in cultured cells have been described in detail [50]. In our study, after a single BrdU/FdU injection, most cells showing the typical S-phase distribution of PCNA were also BrdU-positive, confirming that BrdU had reached all S-phase cells. Moreover, no mutagenic or toxic effects of BrdU [31,39] were detected in animals injected with the BrdU/FdU mixture.

This protocol enabled the study of the relationship between DNA replication zones and PCNA expression in fixed tissues. Our results with single BrdU injection agree with previous studies in vitro demonstrating that PCNA is recruited in active replication sites [9,50].

The number of RMS cells that either undergo cell cycle progression or withdraw from it can be estimated by employing propidium iodide (PI) combined with PCNA detection. Thus, the proliferation frequency in the RMS or similar neurogenerative zones can be determined. The present protocol allows both the proliferation frequency and the survival of RMS cells to be analyzed in the same section by combining PCNA and BrdU detections. Different BrdU administration protocols could be applied to study the survival of progenitor cells of the RMS in the olfactory bulb, analyzing the number of BrdU-positive cells present in the olfactory bulb when the animals are sacrificed at different times after BrdU administration [57]. Furthermore, the cell cycle length of dividing cells (including RMS cells) has been deduced using a protocol of BrdU injection to cumulatively label nuclei undergoing DNA synthesis at replication sites [43,49].

6.2. Trouble-shooting

Although Bouin-4% is an excellent fixative for detecting PCNA and BrdU, its use may be problematic in the detection of other antigens or when performing general histological stains. For instance, Bouin-4% fixation prevents the proper labeling of nucleic acids with chemicals such as PI. PI stains cytoplasm and nucleolus when conventional fixatives (such as buffered paraformaldehyde) are employed, whereas the cytoplasmic stain, but not the nuclear one, is abolished when Bouin-4% and HCl treatments are implemented.

6.3. Alternative and support protocols

Denaturation of DNA is an essential step for the detection of BrdU, HCl being the agent most commonly used [25]. Different HCl concentrations were assayed: 1.5, 2, 2.5 and 3 N. 2 N HCl produced the best results, while a concentration of 3 N was ineffective. A 30 min incubation with 2 N HCl at 37 °C was also tested, resulting in a weaker staining of BrdU, as compared with the proposed 1 h. Microwave treatment of tissues immersed in 0.1 M citrate buffer (pH 6.0) has previously been demonstrated to be effective to allow BrdU detection without using a denaturation step [18]. We tested this treatment but it proved to be ineffective.

Tissues fixed with buffered 4%-paraformaldehyde or with Somogy’s fixative [51] without glutaraldehyde can be used to carry out the double detection of BrdU and PCNA. However, these fixatives have some drawbacks: (a) the required postfixation time is about 3 h, (b) PCNA detection is less precise and (c) non-proliferating cells are also labeled. To solve these problems, free-floating sections can be incubated with Bouin-4% for 2 h before carrying out the double-immunofluorescence detection.

7. Essential literature references

Original papers: [9,16,20,41]
Reviews: [22,47]

8. Quick procedure

I. Perfuse animals with Bouin-4%.
II. Obtain sections with a freezing microtome.
III. Wash tissue 3 × 10 min in PBS.
IV. Incubate tissue in 0.13 M NaBH₄ for 20 min at room temperature.
V. Wash tissue 3 × 10 min in PBS.
VI. Incubate slices in 2 N HCl for 1 h at 37 °C.
VII. Wash tissues in 0.1 M borate buffer (pH 8.5) 3 × 10 min.
VIII. Rinse sections 3 × 10 min in PBS.
IX. Incubate tissue for 48 h at 4 °C in antiserum made up of rat anti-BrdU (1:5000), mouse anti-PCNA (1:3000), 0.2% (w/v) Triton X-100 and 5% (v/v) normal goat serum diluted in PBS.
X. Wash sections in PBS 3 × 10 min.
XI. Incubate tissue in the dark at room temperature for 2 h in fluorochrome antiserum made up of Cy2-conjugated anti-rat IgG antibody (1:200), Cy3-conjugated anti-mouse IgG antibody (1:200) and 0.2% Triton X-100 diluted in PBS.
XII. Wash in PBS 3 × 10 min in the dark.
XIII. Mount with anti-fading medium.

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