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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.

Theme: Development and regeneration
Topic: Cell differentiation and migration

Keywords: Adult neurogenesis; Cell proliferation; Neural migration; Rostral migratory stream; S-phase stage

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

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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 δ [10,46]. The half-life of PCNA is about 20 h, and its expression begins to increase during the late G_1 and early S-phases and declines throughout G_2 and mitosis [27,32,33]. PCNA has been described to be present in some proliferating cells even at G_0, 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-Fluoride-2′-deoxyuridine (#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)