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Dual BrdU-PCNA immunodetection of proliferative cells in dental and orofacial tissues of teleosts

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Summary

We have developed a protocol for simultaneous detection of BrdU and PCNA on paraffin sections of teleost tissues and have tested it successfully on dental and orofacial tissues of Atlantic salmon (*Salmo salar*) and zebrafish (*Danio rerio*). By combining decalcification of the tissue, appropriate antigen retrieval steps, and high quality tissue sections, this protocol allows to study the instantaneous pattern of proliferating cells, as well as cell tracing in large specimens. The technique is therefore highly suitable to study skeletal and dental tissues, tissues that are traditionally not easily accessible to molecular and cellular techniques. Dual BrdU-PCNA immunostaining may serve as a useful tool in experimental studies on fish developmental or regenerative processes such as tissue turnover in orofacial epithelia, continuous tooth replacement, and in the identification of putative stem cells for which the cycling state of BrdU label retaining cells needs to be determined.

Introduction

Cell proliferation studies have been used extensively to reveal mitoses within cell populations, to study cell lineage, and to identify putative stem cells (Plickert and Kroiher, 1988; Leung et al., 2005; Paulus and Müller, 2006; Capuco, 2007; Handrigan et al., 2010). Proliferating cell nuclear antigen (PCNA), a 36KD auxiliary protein of DNA-polymerase δ (Bravo and Macdonald-Bravo, 1987), and the thymidine analogue 5-Bromo-2′-deoxyuridine (BrdU) are frequently used in these types of studies as an endogenous and exogenous marker for cell proliferation, respectively. BrdU replaces thymidine in the DNA-synthesis of the cell cycle (S-phase) whereas the PCNA concentration fluctuates throughout the cell cycle with a maximum concentration in the S-phase (Celis and Celis, 1985). A protocol for dual immunohistological staining of these two markers is a valuable tool and of fundamental importance to know the proliferative state of cells for instance when assessing the cycling capacity of long term labelled stem cells (Harada et al., 1999; Handrigan et al., 2010; Ishikawa et al., 2010). The simultaneous detection of BrdU and PCNA has already been performed in zebrafish (*Danio rerio*) on dissected brain tissue, both as whole mount staining and on cryo-sections (Grandel et al., 2006; Kaslin et al., 2009). In this study we have developed a protocol for the simultaneous detection of BrdU and PCNA on paraffin sections, that can be applied on (i) decalcified tissues of different Teleost species, allowing the study of skeletal and dental tissues also in (large) juvenile and adult specimens, and (ii) different species of teleosts of widely divergent adult size. Moreover, the use of paraffin sections allows the analysis of both juvenile and adult fish tissues at a higher resolution than is possible with cryosections. Here, we illustrate the protocol with dental and orofacial tissues of Atlantic salmon (*Salmo salar*) and zebrafish (*Danio rerio*).

Materials and methods

Animals

A total of 48 Atlantic salmon (*Salmo salar*) (parr stage of the life cycle) with an average weight of 10 g were used for BrdU administration. They were reared in freshwater at a temperature of 13°C. The fish were obtained, handled and sacrificed under license and with support of the Havforskningsinstituttet (Institute of Marine Research, IMR) Matre, Norway. Furthermore, 30 juvenile zebrafish (38 days post-fertilisation, dpf) with an average notochordal length of 7.8 mm were used for BrdU administration. They were bred and maintained at the Ghent zebrafish facility in accordance to Westerfield (1993), and processed in accordance to the Belgian law on the protection of laboratory animals (KB dd. Nov. 14, 1993).

BrdU administration

The Atlantic salmon were injected intraperitoneally with 100 μl (10 μl/g body weight) of a solution containing 10 mg/ml 5-Bromo-2′-deoxyuridine (32.6 mM) (BrdU, Sigma-Aldrich, St. Louis, MO) in 0.1 M phosphate-buffered saline (PBS, pH 7.2). The injection was repeated 5 times every 12 hours for each fish, in an attempt to also label cycling cells which were not in the S-phase of their cell cycle during the first injection. Prior to manipulation, all fish were anaesthetized (Finquel [Argent Chemical Laboratories, Redmond, WA], 100 mg/l). Sampling of the fish occurred at different time points: 4 hours past the last BrdU injection as a pulse, and 1, 2, 4 and 8 weeks past the last BrdU injection as chase time. For the present study we focused on animals having received a 2-week chase period.

Given their small size, zebrafish were incubated in 0.5% BrdU solution (16.3 mM) in E3-medium (Westerfield, 1993) for 2 hours, and allowed to recover in E3-medium for 6 hours. This was repeated three times. The experimental set-up was analogous to that used for Atlantic salmon: fish were sampled immediately after the last BrdU incubation as well as after 5, 10, 15 and 20 days after the last incubation. For the present study we focused both on animals sacrificed immediately after the last incubation as well as after a chase time of 5 days.

Tissue fixation, decalcification and sectioning

The Atlantic salmon were anaesthetized (Finquel, 100 mg/l) and killed by decapitation. Lower jaws were dissected and fixed in 4% paraformaldehyde (PFA, pH 7.2) at 4°C for 48 hrs. The jaws were rinsed three times in 0.1 M PBS and decalcified for at least 3 weeks in Morse solution: 22.5% formic acid, 10% sodium citrate (Morse, 1945). The juvenile zebrafish were killed by an overdose of anaesthesia (MS-222, 10 mg/l), fixed in 4% paraformaldehyde, but not decalcified. Tissues of both species were embedded in paraffin and sectioned at 5 μm (Microm HM360, Prosan, Merelbeke, Belgium).

Immunohistochemistry

Single immunohistological staining protocols for PCNA and BrdU were adapted from Lema et al. (2005), Ortego et al. (1994) and Shimada et al. (2008).

Sections were heated on a hotplate at 70°C for 5 minutes (min.), allowed to cool down for 5 min. and heated again for 5 min., before submersion in a parasolve bath for 25 min. in total (changed once after 15 min.). The deparaffinized sections were rehydrated in a graded series of ethanol and rinsed three times in PBS-D (PBS 0.1 M/dimethyl sulfoxide, DMSO 1%). After a short wash in distilled water, sections of Atlantic salmon were submerged in 0.1 M $ZnSO_4$ (pH 5.45). These sections were heated in the microwave for 3 min., allowed to cool down for 1 min. in distilled water and heated again for 3 minutes in the $ZnSO_4$ buffer. The heating procedure was performed at 1000 W and time was counted as soon as the buffer started to boil. Next, sections were rinsed in distilled water and allowed to cool for 20 min. Sections of zebrafish were submerged in preheated TRIS/EDTA buffer (pH 9.95°C) and heated in a 95°C water bath for 20 min. They were next allowed to cool down in the same buffer for 1 hour. These antigen retrieval steps are crucial to expose antigenic sites/epitopes to the anti-PCNA antibody. All further steps were similar for both species. Sections were rinsed in PBS-D, followed by chromatin precipitation in 2 M HCl for 30 min. The reaction was stopped by 5 min. incubation in 0.1 M sodium tetraborate. Sections were washed with PBS-D and submerged in blocking solution (3%BSA, 1%Milk powder in PBS-D) for 2 hours. The primary anti-BrdU antibody (monoclonal Anti-BrdU antibody produced in Rat (IgG), ab6326, Abcam) was diluted 1/100 in blocking solution, and applied overnight at 4°C. Subsequently sections were rinsed three times for 5 min. with PBS-D and a secondary anti-rat antibody (Alexa fluor 488, goat anti-rat IgG, a11006, Invitrogen, Merelbeke, Belgium), diluted 1/200 in blocking solution, was applied for 1 hour. To prevent bleaching of the fluorescent signal, sections were kept in the dark for the rest of the protocol. Sections were rinsed again for three times in PBS-D and an anti-PCNA monoclonal antibody produced in mouse (P8825, Sigma, St. Louis, MO), diluted 1/100 in blocking solution, was applied overnight at 4°C. Sections were rinsed three times in PBS-D and a secondary anti-mouse antibody (Alexa Fluor 555, a21422, Invitrogen), diluted 1/200 in blocking solution, was applied for 1 hour. We used fluorescent secondary antibodies, given that often high amounts of pigment can obscure unequivocal identification of proliferating cells when using avidin-biotin methods. Sections were subsequently rinsed in PBS-D and counterstained with DAPI (1/1000 dilution of 1 μg/ml stock solution) (Invitrogen, Carlsbad, USA). Control sections were incubated in the blocking solution without the two primary antibodies and received the secondary antibodies only. After final rinsing, slides were mounted with Vectashield (Vector laboratories Inc., Burlingame, USA). Labelling was analyzed using an epifluorescence microscope (AXIO Imager Z1, Zeiss, Göttingen, Germany). Photographs were taken using an Axiocam MRc videocamera and AXIOVISION release 4.8 software (Zeiss MicroImaging, Jena, Germany).

Results

Immunodetection of PCNA in zebrafish sections was achieved using TRIS/EDTA buffer in a 95°C water bath. For Atlantic salmon, this long term heating caused severe tissue damage, likely due to the larger size of the sections (data not shown). In our hands antigen retrieval in Atlantic salmon by means of heating in $ZnSO_4$ buffer, produced superior results for immunohistological PCNA detection (modified from Ortego et al., 1994).

Sections through the dentigerous region of the lower jaw in Atlantic salmon or the pharyngeal jaws in zebrafish showed numerous labelled cells in the different dental tissues (Fig. 1). The dual BrdU-PCNA detection allowed to distinguish between cells that are BrdU-positive (green), PCNA-positive (red) and positive for both (red/green overlap, Fig. 1C). Because a chase time was allowed after BrdU injection, PCNA and BrdU displayed different labelling patterns, BrdU-positive cells having undergone DNA synthesis at the time of BrdU injection, and PCNA-positive cells preparing for mitosis at the time of fixation. For example, in Atlantic salmon, PCNA-positive cells within a tooth family were observed at the periphery of the epithelial dental organ whereas BrdU was localized more to the central region of the epithelial dental organ, as well as in the odontoblasts (compare Fig. 1A to 1B). In contrast, dual PCNA and BrdU immunodetection in zebrafish sampled immediately after the last BrdU incubation, showed comparable labelling patterns for both markers (compare Fig. 1D and 1E). However, some cells displayed PCNA label in the absence of BrdU signal, which might be an indication for a higher labelling index for PCNA.

In the jaw epithelium of Atlantic salmon (2 weeks chase period, Fig. 2A,B) and the skin of zebrafish (5 days chase period, Fig. 2C,D) labelling patterns again differed for PCNA and BrdU. In the thick orofacial epithelium of Atlantic salmon, for example, most BrdU-positive cells are situated in the most superficial layers of the epithelium. The majority of the cells in the middle layers show a scattered label in their nucleus. Few labelled cells are present in the stratum basale. In contrast, PCNA-positive cells are most prominently situated in and near the stratum basale and middle region of the epithelium, and labelling is more homogenous within the nuclei.

Discussion

In this paper we have successfully applied a protocol for simultaneous immunohistological detection of BrdU and PCNA in tissue sections of different teleost species. By combining decalcification of the tissue, appropriate antigen retrieval steps, and high quality tissue sections, this protocol allows to study the instantaneous pattern of proliferating cells, as well as cell tracing in large specimens.

The protocol that we present is identical for salmon and zebrafish tissues, with the exception of the antigen retrieval step. In our hands, short heating performed in the microwave, rather than long baths, yielded the best results for the salmon sections.

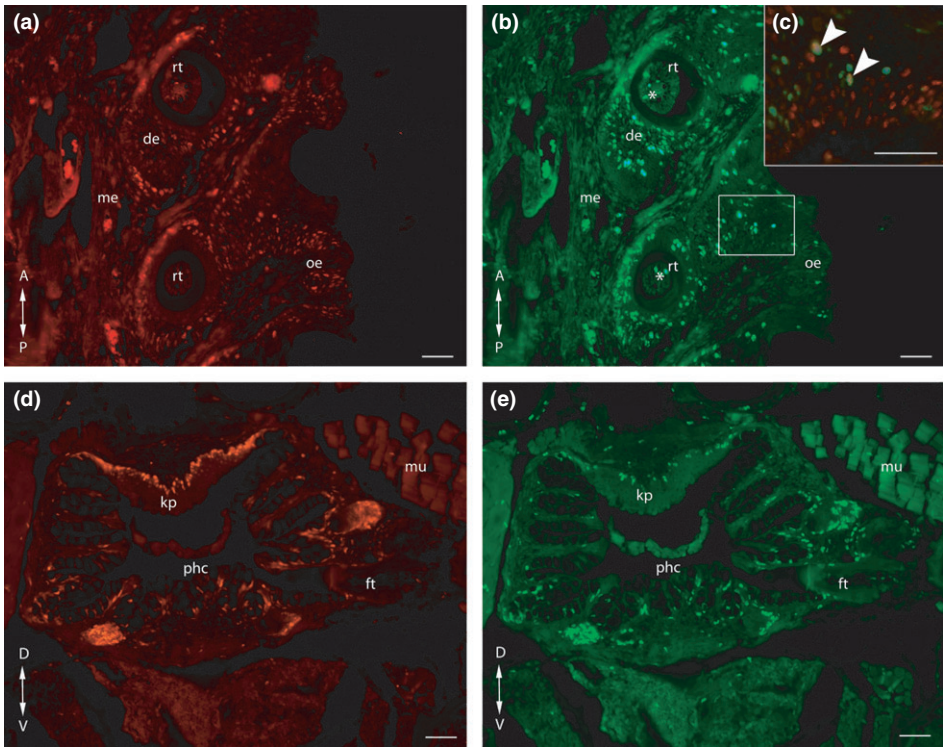

Fig. 1. (a, b) Longitudinal section through the lower jaw of Atlantic salmon (two-week chase period) showing a number of tooth germs. (c) Higher magnification of the boxed area in Fig 1b, with an overlay of BrdU and PCNA immunolabelling. Note single PCNA-labelled cells, single BrdU-labelled cells and double-labelled cells (arrowheads). (d, e) Transverse section through the head of zebrafish (pulse period) at the level of the pharyngeal jaws, showing a number of tooth germs at various stages of development. Note, e.g., the lower amount of BrdU- compared to PCNA-labelled cells in the basal layer of the keratinized pad covering the roof of the pharyngeal cavity. Immunodetection of PCNA in red (a, c, d), immunodetection of BrdU in green (b, c, e). Asterisks show differentiated odontoblasts in the pulp cavity of a replacement tooth. a, anterior; d, dorsal; de, dental epithelium; ft, functional tooth; kp, keratinized pad; me, mesenchyme; mu, muscle; oe, oral epithelium; P, posterior; phc, pharyngeal cavity; rt, replacement tooth; V, ventral. Scale bar in a–e = 50 μm

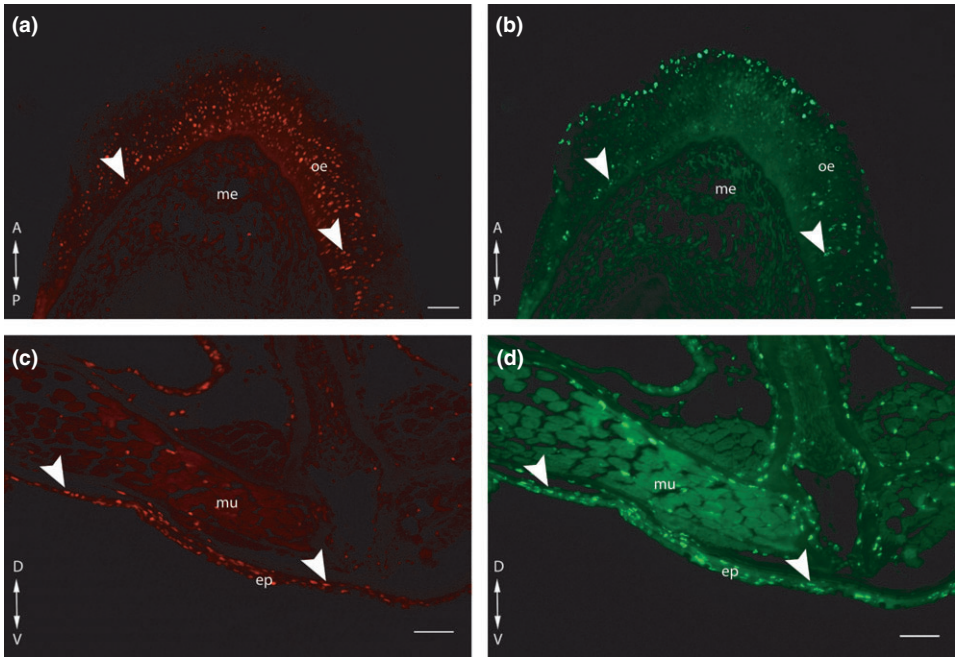

Fig. 2. (a, b) Longitudinal section through the lower jaw of Atlantic salmon (two-week chase period). Note BrdU-labelled cells in the superficial layers of the oral epithelium, and PCNA labelled cells near the basal and middle region of the oral epithelium. Double BrdU-PCNA stained cells are located predominantly near the basal layer of the orofacial epithelium, i.e., the putative location of progenitor cells responsible for tissue renewal. (c, d) Transverse section through the head region of a zebrafish (5 days chase period) with labelled skin epithelium at the ventral side of the sternohyoideus muscle. Immunodetection of PCNA in red (a, c), immunodetection of BrdU in green (b, d). Arrowheads show double BrdU-PCNA labelled cells. a, anterior; d, dorsal; ep, skin epithelium; me, mesenchyme; mu, muscle; oe, oral epithelium; P, posterior; V, ventral. Scale bar in a–d = 50 μm

This is probably due to the large size of the salmon sections and thus the more delicate adhesion of the tissues to the slides. Extending the protocol to other teleost species or other vertebrates in general may require testing appropriate steps for antigen retrieval specific for the species and / or tissue under investigation. Thus, different antigen retrieval buffers such as $ZnSO_4$, citrate and TRIS / EDTA followed by retrieval methods, should be tested on each new species, as this appears to be the most critical step of the protocol. Nonetheless, the protocol can be applied regardless of whether the tissue was decalcified or which mode of BrdU administration was used. While this suggests a considerable flexibility, the specificity of the protocol appears to be guaranteed, as shown by the expected location of proliferating cells in the tissues considered.

Labelling indices derived from BrdU and PCNA staining are not necessarily similar, as shown by Connolly and Bogdanffy (1993). Even when tissues are fixed immediately after a BrdU pulse (i.e., without chase time), the pattern of BrdU- and PCNA-labelled cells is different. This can be ascribed to the nature of these proliferation markers. BrdU labels cells in the S-phase of the cell cycle, while PCNA labels cells from the early G1 to the late S-phase (Celis and Celis, 1985; Connolly and Bogdanffy, 1993; Sasaki et al., 1994; Garrett and Guthrie, 1998). When chase times are used, the difference between the two labelled cell populations increases even more, as cells having incorporated BrdU can divide one or multiple times, migrate, undergo apoptosis etc., prior to tissue fixation. The use of BrdU immunostaining combined with PCNA at the end of a chase time distinguishes cells that have stopped proliferation (terminally differentiated cells, possibly having exited the cell cycle) from cells that are still cycling. The former contain BrdU label but do not stain with PCNA, the latter are double labelled. Such a distinction is of prime importance in studies focusing on label retaining cells and the potential stemness of such cells (Cotsarelis et al., 1990; Harada et al., 1999; Morris and Potten, 1999; Handrigan et al., 2010; Ishikawa et al., 2010).

A dual BrdU-PCNA staining has been used previously on whole mount tissue as well as on 14 μm thick cryosections of dissected zebrafish brains (Grandel et al., 2006; Kaslin et al., 2009). In these studies the focus was on one specific type of tissue in only one species. The strength of the protocol presented here is that it uses thin (5 μm) sections prepared from decalcified tissues of large specimens. This allows it to be used for example on adult skeletal and dental tissues, tissues that are traditionally more difficult to access with molecular or cellular techniques. More specifically, the resolution provided by the use of 5 μm sections should facilitate studies addressing cell proliferation and cell dynamics, where cell counts and the topology of the cells within a 3D tissue context are important. In addition, the high resolution allows for the precise localisation of BrdU label retaining cells, often considered a hallmark of adult stem cells (Harada et al., 1999; Barker et al., 2007; Jaks et al., 2008; Handrigan et al., 2010).

Current experiments in our lab are focusing on mechanisms of continuous tooth renewal in teleost fish. The protocol presented here will be an important asset to identify the possible existence of label retaining cells involved in tooth replacement that are not terminally differentiated, and thus retain potential stemness.

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