

Detection of Bromodeoxyuridine in Paraffin-embedded Tissue Sections Using Microwave Antigen Retrieval is Dependent on the Mode of Tissue Fixation

WESLEY M. GARRETT and H. D. GUTHRIE

Germplasm and Gamete Physiology Laboratory, Agricultural Research Service, United States
Department of Agriculture, Beltsville, MD 20705

Key Words

proliferative activity, detection of Bromodeoxyuridine (BrdU), tissue fixation, immunostaining

Abstract

A simple routine microwave antigen retrieval procedure allows the sensitive detection of incorporated BrdU in pulse labeled cells. Of the two fixatives tested, Carnoy's offers superior nuclear morphology, but with a sacrifice of immunostaining intensity. For investigations where animals are sacrificed within several hours after pulse labeling, Carnoy's fixative may prove adequate as a general fixative, but it is not known what effect it has on cellular antigens of interest. For our purposes, 10% neutral buffered formalin was found to be a superior fixative because of its ability to crosslink nuclear proteins and associated chromatin, resulting in more intense immunostaining for BrdU. In addition, we have found that formalin fixation coupled with microwave antigen retrieval is completely compatible with immunostaining of other antigens of interest.

Introduction

Bromodeoxyuridine (BrdU), an analog of thymidine, is incorporated into DNA during the S-phase of the cell cycle, and is a useful alternative to labeling proliferating cells with tritiated thymidine. Incorporated BrdU is detected immunologically with the use of monoclonal antibodies, and the resulting labeled cells can be analyzed either by flow cytometry, or microscopy. Since BrdU is detected immunologically, labeled cells can be observed within hours. In contrast, cells labeled with tritiated thymidine must be detected by autoradiography which can take weeks to months. Because BrdU is incorporated into proliferating cells, its use includes estimates of cell cycle kinetics, growth fractions, and the proliferative status of tissues. To study the proliferation potential of specific tissues, animals are injected with BrdU, sacrificed one or two hours later, and the tissues removed and fixed for microscopic analysis. A common fixative used for BrdU labeling is Carnoy's, which is a mixture of acetic acid, alcohol, and chloroform [1]. To detect incorporated BrdU immunologically, the DNA must be denatured to allow antibody access. Denaturation of DNA in Carnoy's fixed samples is usually accomplished by treatment with HCl. Stronger crosslinking fixatives such as formalin or glutaraldehyde can inhibit detection of BrdU, requiring a combination of enzymatic treatment with pepsin in addition to DNA denaturation with HCl [1]. As an alternative for enzymatic digestion, microwave antigen retrieval can be used to unmask antigens of interest in tissues that have been fixed in formalin and embedded in paraffin [2], and this technique has been used successfully to detect BrdU in long-term labeled cells [3].

The proliferative activity of ovarian follicles in farm animals has been investigated using BrdU labeling followed by tissue fixation in Carnoy's fixative [4-5]. We were interested in determining the proliferative status of pig ovarian follicles during the luteal phase using BrdU pulse labeling. We also wanted to use consecutive tissue sections for immunostaining other antigens of interest. The immunostaining procedures we had used previously involved either frozen sections, or formalin-fixed paraffin sections that had been subjected to microwave antigen retrieval. While Carnoy's appeared to be the fixative of choice for BrdU-labeled tissues, we did not want to have to revalidate our immunostaining procedures in Carnoy's-fixed, paraffin-embedded ovarian tissue. For this reason, we set out to test the efficacy of labeling pig ovarian follicles with BrdU, comparing fixation in formalin with fixation in Carnoy's. In accordance with our procedure for immunostaining paraffin sections, microwave antigen retrieval would be used for unmasking the incorporated BrdU.

Materials and Methods

Reagent preparation

Bromodeoxyuridine was dissolved in sterile saline at a concentration of 15 mg/ml immediately before use. For tissue fixation, a solution of 10% neutral buffered formalin was prepared by diluting full strength commercial formalin (37% formaldehyde) 1:10 in phosphate buffered saline. Carnoy's fixative was prepared from ethanol, chloroform, and glacial acetic acid at a ratio of 6:3:1. Antigen retrieval buffer consisted of a 10 mM solution of sodium citrate adjusted to pH 6 with concentrated HCl.

Injection of BrdU and tissue fixation

In our initial test, BrdU was injected intravenously into the jugular vein of a female pig on day 3 of the estrous cycle. A dose of 5 mg/kg was chosen, as this concentration had been used successfully in sheep and pigs to label S-phase cells in ovarian follicles [4–5]. At sacrifice two hours later, the ovaries were excised, and immersion-fixed for 24 h in either 10% neutral buffered formalin, or Carnoy's fixative. The fixed ovaries were then transferred to 70% ethanol until they were processed for paraffin embedding.

Preparation of tissues for BrdU immunohistochemistry

Blocks of ovarian tissue were sectioned at a thickness of 5 μ m on a Leitz 1512 rotary microtome, and the sections were stretched on a water bath, then collected on Superfrost® Plus precoated microscope slides. The slides were dried overnight at 37°C, then baked at 56°C for 30 min to adhere the sections firmly to the slides. The sections were routinely deparaffinized in xylene, and rehydrated through a descending alcohol series, then rinsed 3x 2 min in deionized water. At this point, the slides were transferred to a plastic staining rack and immersed in approximately 350 to 400 ml of 10 mM citrate buffer (pH 6) in a glass staining dish, with the lid in place. Microwave antigen retrieval was carried out in a General Electric microwave oven rated at 700 Watts. The dish was placed in the middle of the microwave oven, and heated for five minutes at full power. Following a five minute rest period, the slides were again heated for an additional five minutes at full power. The citrate solution

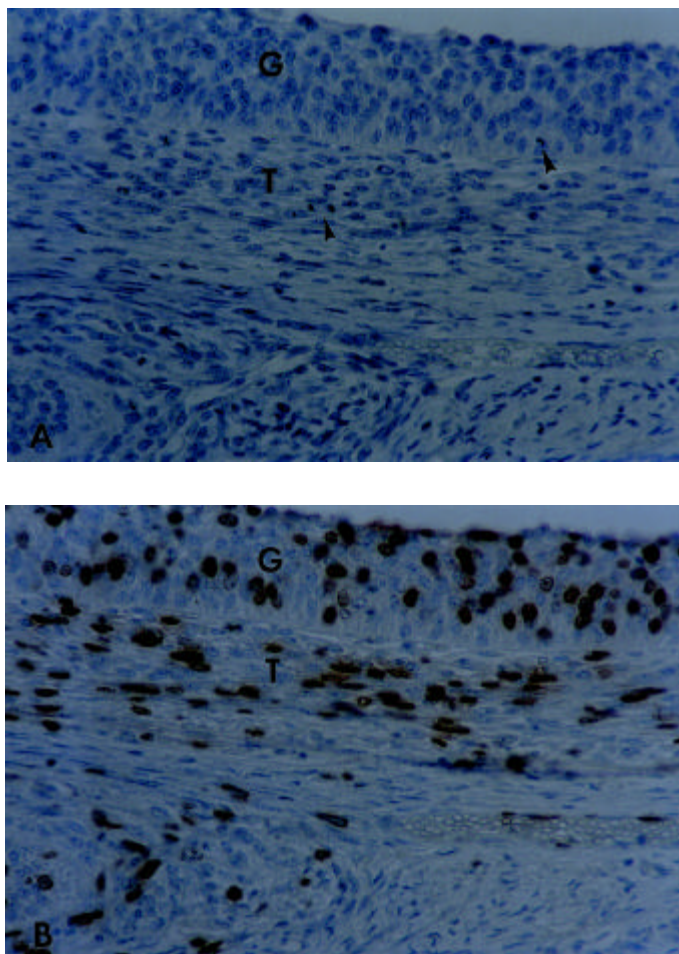


Figure 1 Small ovarian follicle from an ovary fixed in Carnoy's and treated with 4 μ g/ml mouse-IgG (A) or with 1 μ g/ml anti-BrdU (B). Both sections are counterstained with hematoxylin. Note the fine, granular, nuclear structure revealed with this fixative. Two mitotic figures are represented by arrowheads in the negative control section (A). The brown-stained nuclei in both the granulosa cell (G) and thecal cell (T) layers in (B) incorporated BrdU during the 2 h *in vivo* labeling period, and have been detected with the anti-BrdU monoclonal antibody following microwave antigen retrieval.

began to boil after about four minutes in the first round of heating, and boiled continuously during the second round. We did not find it necessary to add more solution during the antigen retrieval procedure, because the staining dish lid prevented significant evaporation during the heating phase, and the initial volume of buffer was sufficient to cover the slides by 3/4 of an inch. Once the heating was finished, the dish was removed from the microwave oven, and the slides were left in the hot citrate buffer for an additional 30 min. The slides were then rinsed for five min in 50 mM Tris buffered saline (TBS) pH 7.6 in preparation for immunostaining.

Immunostaining procedure

Incorporated BrdU was detected with a mouse monoclonal antibody (Clone BMC 9318) and visualized by the Peroxidase, Anti-Peroxidase (PAP) technique. The immunostaining procedure was modeled according to our procedure used for frozen sections [6]. In brief, non-specific staining of sections was reduced by blocking for 30 min with 5% normal goat serum (NGS) in TBS. Following the blocking step, the sections were incubated with anti-BrdU at either 1, 2, or 4 μ g/ml in TBS 1% NGS overnight in a humidified chamber at 4°C. The following day, the slides were rinsed in TBS (3x 5 min), and treated for 30 min with

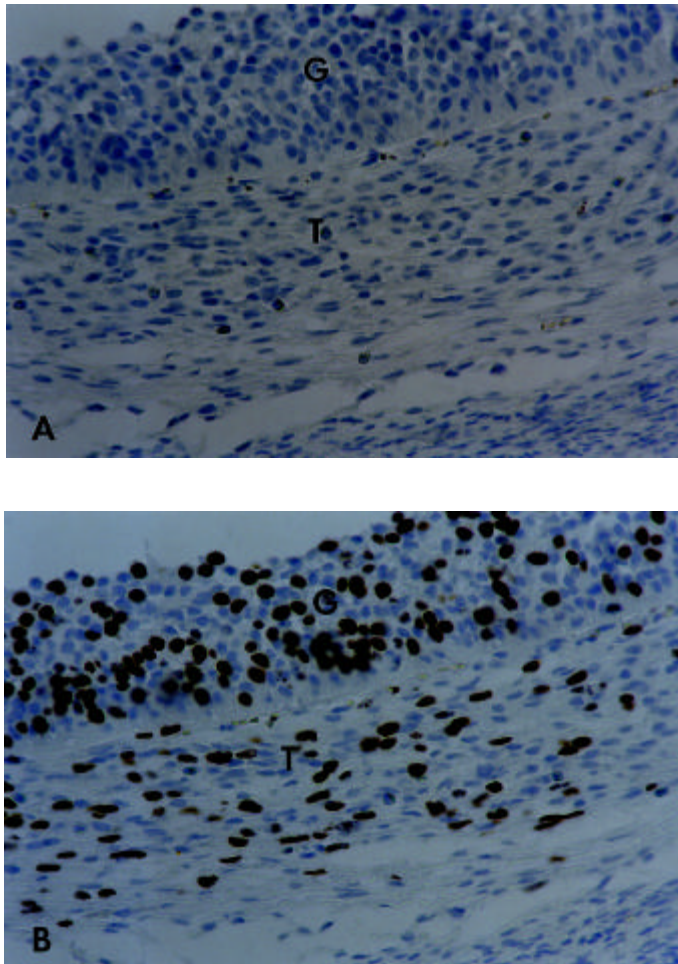


Figure 2 Small ovarian follicle from an ovary fixed in neutral buffered formalin and treated with 4 $\mu\text{g/ml}$ mouse IgG (A) or with 1 $\mu\text{g/ml}$ anti-BrdU (B). Both sections are counterstained with hematoxylin. Note that the fine, granular nuclear structure observed in the Carnoy's-fixed sample in Figure 1 is absent, and the cell nuclei stain a uniform blue color with hematoxylin. Mitotic figures are not readily distinguishable. The brown-stained nuclei in both the granulosa cell (G) and thecal cell (T) layers in (B) incorporated BrdU during the 2 h *in vivo* labeling period, and have been detected with the anti-BrdU monoclonal antibody following microwave antigen retrieval. Note how these nuclei are more densely stained than the corresponding Carnoy's-fixed sample in Figure 1B.

unlabeled goat anti-mouse IgG diluted 1:200 in TBS 1% NGS plus 2% pig serum. The slides were again rinsed with TBS (3x 5 min), and then treated for 30 min with mouse peroxidase anti-peroxidase complex diluted 1:200 in TBS 1% NGS. Following rinses in TBS, (3x 5 min) final color development was accomplished by treating the slides with 0.05% diaminobenzidine hydrochloride (DAB) and 0.02% hydrogen peroxide in TBS for 8 min. This reaction was terminated by washing the slides in deionized water. The slides were counterstained with Gill's hematoxylin #2 diluted 1:1 with water, then dehydrated in ascending alcohols, cleared in xylene, and glass

cover slips were mounted with Permaslip. Negative control slides were prepared by substitution of mouse IgG for the primary antibody.

Results

Influence of fixative on tissue morphology

Both fixatives were found to adequately penetrate and fix the pig ovarian tissue, but there were notable differences in the staining characteristics with the two fixatives. Upon staining with hematoxylin, sections from Carnoy's fixed ovary exhibited a fine nuclear structure which allowed the discrimination of both light staining, dis-

persed euchromatin, and darkly stained specks of condensed heterochromatin (see Figure 1A). Numerous mitotic figures could be seen in both the granulosa and thecal cells of ovarian follicles. The cell nuclei of samples fixed in buffered formalin stained a uniform, dense blue color with hematoxylin, with no distinction between dispersed and condensed chromatin structure (see Figure 2A). Mitotic figures could not be localized in the formalin fixed ovary.

Influence of fixative on immunolocalization of BrdU

With the use of microwave antigen retrieval, BrdU was easily detected in S-phase cells in both the granulosa and thecal cell layers of growing ovarian follicles. As we had seen with hematoxylin staining, there was a marked difference in immunostaining for BrdU in Carnoy's versus formalin-fixed tissue. While BrdU was detectable in Carnoy's fixed tissues at all doses of antibody tested, the staining pattern mimicked that seen with hematoxylin (i.e., labeled cell nuclei exhibited a diffuse brown stain at a dose of 1 $\mu\text{g/ml}$ of antibody) (see Figure 1B). In contrast, immunostaining for BrdU in formalin fixed tissue resulted in very dense, dark brown staining of labeled nuclei at a dose of 1 $\mu\text{g/ml}$ of antibody (see Figure 2B).

Discussion

This study was performed to assess the feasibility of labeling follicles during the luteal phase of the pig estrus cycle with BrdU. In order to accomplish this goal, we had to decide on a suitable fixative which would allow both adequate morphological preservation and strong immunostaining, and develop an antigen retrieval method that was routine, sensitive, and compatible with our other immunostaining procedures. We had previously tested microwave antigen retrieval of formalin-fixed, paraffin embedded tissue sections for the immunohistochemical analysis of steroidogenic enzymes and cell proliferation antigens. The results indicated that this method of unmasking antigens was extremely sensitive, in some cases more sensitive than immunohistochemistry on frozen sections. In the previous studies of BrdU labeling of ovarian follicles in sheep and pigs [4-5], Carnoy's fixative had been used for tissue

fixation, and for this reason, we tested this fixative alongside buffered formalin in order to determine the best choice for our study. Our preference was formalin since we had already validated our other immunohistochemical procedures with this fixative in paraffin embedded tissue. We found that there was a vast difference in nuclear morphology between the two fixatives. While Carnoy's fixative allowed discrimination of fine nuclear structure, and in this regard was superior to buffered formalin, the staining intensity for BrdU was significantly less than with buffered formalin. The two fixatives actions on proteins are different; Carnoy's fixative precipitates proteins whereas formalin forms crosslinks between amino groups. We have assumed, that the crosslinking ability of formalin allows greater retention of nuclear proteins and associated chromatin, thus retaining more BrdU for immunohistochemical detection. This crosslinking is what can cause the masking of antigens of interest, thus obviating some method of antigen retrieval.

In order to detect incorporated BrdU in tissue sections, the DNA requires denaturation. Depending on the mode of fixation, this is usually accomplished by acid treatment, or a combination of acid treatment and enzymatic digestion if crosslinking fixatives are used (1). The disadvantage of these methods is that they are more time-consuming than microwave antigen retrieval, and they can also destroy other antigens of interest, thus eliminating the potential for multiple labeling studies. A recent report has shown the feasibility of triple immunostaining for BrdU and catecholamine-synthesizing enzymes in formalin-fixed, paraffin-embedded tissues with the use of microwave antigen retrieval (3). The author reported that immunohistochemistry following microwave antigen retrieval yielded specimens of excellent quality that were not obtainable with the conventional method of DNA denaturation using pepsin digestion and HCl treatment.

In summary, we have found that a simple routine microwave antigen retrieval procedure allows the sensitive detection of incorporated BrdU in pulse labeled cells. Of the two fixatives tested, Carnoy's offers superior nuclear morphology, but with a sacrifice of immunostaining intensity. For investigations where animals are sacrificed

within several hours after pulse labeling, Carnoy's fixative may prove adequate for a general fixative, but it is not known what effect it has on cellular antigens of interest. For our purposes, 10% neutral buffered formalin was found to be a superior fixative, because of its ability to crosslink nuclear proteins and associated chromatin, resulting in more intense immunostaining for BrdU. In addition, we have found that formalin fixation coupled with microwave antigen retrieval is completely compatible with immunostaining of other antigens of interest.

References

1. Schutte, B., Reynders, M.M.J., Bosman, F.T., and Blijham, G.H. (1987) Effect of tissue fixation on anti-bromodeoxyuridine immunohistochemistry. *J. Histochem. Cytochem.* **35**:1343-1345.
2. Shi, S.-R., Key, M.E., and Kalra, K.L. (1991) Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave heating of tissue sections. *J. Histochem. Cytochem.* **39**:741-748.
3. Tischler, A.S. (1995) Triple immunohistochemical staining for bromodeoxyuridine and catecholamine biosynthetic enzymes using microwave antigen retrieval. *J. Histochem. Cytochem.* **43**:1-4.
4. Jablonka-Shariff, A., Fricke, P.M., Grazul-Bilska, A.T., Reynolds, L.P., and Redmer, D.A. (1994) Size, number, cellular proliferation, and atresia of gonadotropin-induced follicles in ewes. *Biol. Reprod.* **51**:531-540.
5. Fricke, P.M., Ford, J.J., Reynolds, L.P., and Redmer, D.A. (1996) Growth and cellular proliferation of antral follicles throughout the follicular phase of the estrous cycle in meishan gilts. *Biol. Reprod.* **54**:879-887.
6. Garrett, W.M., Guthrie, H.D. (1996) Expression of androgen receptors and steroidogenic enzymes in relation to follicular growth and atresia following ovulation in pigs. *Biol. Reprod.* **55**:949-955.

Product	Cat. No.	Pack Size
BrdU Labeling and Detection Kit I	1 296 736	100 tests
BrdU Labeling and Detection Kit II	1 299 964	100 tests
BrdU Labeling and Detection Kit III	1 444 611	1000 tests
Anti BrdU-AP, F(ab) ₂ fragments	1 758 748	15 U (1 ml)
PAP Complex (peroxidase-anti-peroxidase)	1 092 626	25 U