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Application of heat-induced antigen retrieval on whole mount immuno-staining for adult mouse cerebellum

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Abstract

The present study examined the application of heat induced antigen retrieval techniques on whole mount immunostaining for the adult mouse cerebellum. After a high-temperature antigen retrieval procedure, cerebella were processed for whole mount immunostaining using a modified protocol developed by Sitolloe and Hawkes (J Histochem Cytochem 50:235-244, 2002). The expression of compartmentation antigens, zebrin II and heat shock protein 25, was analyzed. Characteristic striped patterns of these two antigens were obtained by the present modified protocol. Vibratome sections of whole mount cerebella showed a positive staining in dendrites of particular subsets of Purkinje cells. This optimized protocol circumvents the need to process the cerebella through cycles of chilling and thawing prior to the addition of primary antibody. Thus, heat induced antigen retrieval provides a more rapid and efficient way to perform whole mount immunostaining of adult tissues. This optimized approach allows rapidly and easily for investigation of the spatial distributions of various antigens in adult tissues or organs using whole mount staining.

Introduction

Whole mount immunostaining is a widely used method to visualize the spatial distributions of specific antigens in embryos, fetuses, and dissected organs in whole mount. This method was developed on the basis of immunohistochemistry [1-3]. The mild tissue processing procedure of whole mount immunostaining allows for the preservation of antigenicity in tissues. However, it is difficult to apply such procedures to dense and compact tissues, such as adult brain tissue, because penetration of antibodies into tissues is limited to a depth of 8–9 μm [4]. Sillitoe and Hawkes (2002) have developed a whole mount immunostaining protocol that facilitates analysis of the spatial distributions of cerebellar compartmentation antigens in the adult mouse cerebellum [5]. Using this protocol, antibody penetration extends to at least 250 μm from the pial surface of the cerebellar cortex. Furthermore, this protocol allows for visualization of particular components of the olivocerebellar tracts in whole mount [6]. However, in order to obtain better penetration of the antigens into tissues while maintaining the integrity and morphology of structures, this protocol requires that the cerebella be

passed through 5 cycles of chilling to -80 ^{o}C and thawing at room temperature for 60 min each in 100% methanol prior to primary antibody addition [5]. This adds an additional 10 hr to the total procedure time.

The present study was undertaken to optimize the current whole mount protocol. We have modified whole-mount preparations of cerebellar tissue by adding a heat induced antigen retrieval step prior to primary antibody addition. Antigen retrieval techniques have greatly contributed to the immunohistochemical analysis of formalin-fixed and paraffin embedded materials by recovering cryptic epitopes in the tissues [7]. In this study we show that use of antigen retrieval reduces the procedure time considerably while maintaining the integrity of the tissue sample as observed by expression of the compartmentation antigens, zebrin II and heat shock protein 25 (HSP25).

Materials and Methods

Animals

Male Slc:ICR mice at 7 weeks of ages were purchased from Japan-SLC (Hamamatsu, Japan). In total, 14 male mice

were used in the present study: three each for zebrin II and HSP25 whole mount immunostaining in the original protocol; three each for zebrin II and HSP25 whole mount immunostaining in the protocol with heat induced antigen retrieval; two for section immunohistochemistry. Mice were anesthetized by intraperitoneal injection of trichoroacetaldehyde (400 mg/kg body weight), and were perfused with 0.9% NaCl followed by 4% paraformaldehyde in 0.01 M phosphate buffered saline, pH 7.4 (PBS). The cerebella were stored in the same fixative before use.

Antibodies

Anti-zebrin II (a gift from Prof. R. Hawkes, University of Calgary, Canada), a mouse monoclonal antibody, was produced by immunization with a crude cerebellar homogenate from the weakly electric fish *Apteronotus* [8] and subsequently shown to bind the respiratory isoenzyme aldolase C (Aldoc) [9,10]. It was used directly from spent hybridoma culture medium. Rabbit polyclonal anti-heat shock protein 25 (anti-HSP25), raised against full-length recombinant mouse HSP25 protein, was purchased from StressGen (Victoria, BC, Canada: SPA-801, lot #B111411). It recognizes both the phosphorylated and non-phosphorylated forms of HSP25 and yielded a cerebellar staining pattern identical to that reported previously (Armstrong et al., 2000). Antibody absorption controls, using full-length recombinant mouse HSP25 protein, abolished all immunostaining [11].

Original protocol of whole mount immunostaining

Whole mount immunostaining was performed as follows by using a modified protocol originally designed by Sillitoe and Hawkes (2002) [5].

Step 1: Post-fix in Dent's fixative (ethanol:dimethylsulfoxide (DMSO) = 4:1) overnight at room temperature (RT).
Step 2: Immerse in Dent's bleach (ethanol: DMSO:30% H_2O_2 = 4:1:1) overnight at RT to inactivate endogenous peroxidase.
Step 3: Wash the tissue 3 times with 100% ethanol for 60 min each.
Step 4: Subject the tissue to 5 cycles of chilling to -80 ^oC and thawing at RT in 100% ethanol.
Step 5: Rehydrate the tissue with 50% methanol, 15% methanol and PBS for 90 min each.
Step 6: Enzymatically digest the tissue in 10 μg/ml proteinase K (> 600 units/ml; Boehringer Mannheim Inc., Quebec, Canada) in PBS for 5 min at RT to improve subsequent reagent penetration.
Step 7: Rinse the tissues three times with PBS for 10 min each.
Step 8: Incubate the tissue with PBS containing 2% non-fat skim milk and 0.1% Triton X-100 (PBSMT) overnight at 4 ^oC.
Step 9: Incubate the tissue for 48 hr at 4 ^oC with anti-zebrin II (1:200) or anti-HSP25 (1:500) each diluted in PBS containing 10% normal goat serum (NGS), 0.1% Triton X-100 and 5% DMSO.
Step 10: Rinse the tissues with PBSMT for 10 min each.
Step 11: Incubate the tissue for 24 hr at 4 ^oC with a peroxidase-conjugated anti-mouse IgG (1:200, MBL Co., Ltd., Nagoya, Japan) for zebrin II staining, and a peroxidase conjugated anti-rabbit IgG (1:200, MBL) for HSP25 staining, each diluted in PBSMT containing 5% DMSO.
Step 12: Wash the tissue twice with PBSMT for 2 hr at 4 ^oC.
Step 13: Rinse the tissue with PBS containing 0.2% bovine serum albumin and 0.1% Triton X-100 (PBT) for 2 hr at 4 ^oC.
Step 14: Incubate the tissue with 0.05% DAB and 0.015% H_2O_2 in PBT to visualize the immunoreactive products.

Whole mount immunostaining protocol with heat-induced antigen retrieval

The following heat-induced antigen retrieval procedure was performed. *Steps 3-6* of the original protocol were omitted.

A) Rehydrate the tissue with 50% methanol, 15% methanol and PBS for 90 min each.
B) Treat the tissue with Antigen Retrieval Reagent UNIVERSAL (R&D system, Minneapolis, MN, lot #950512) for 30 min in a 90^oC water bath.
C) Cool the tissue for 30 min at 4 ^oC.

Sectioning of whole mount immunostained cerebella

Following the capture of whole mount photomicrographs, 60 μm vibratome sections of the cerebella were made serially in a transverse plane by using a Microslicer DTK-2000 (Dosaka EM Co. Ltd, Kyoto, Japan).

Section immunohistochemistry

Section immunohistochemistry for zebrin II and HSP25 was conducted to compare the staining patterns with sections of adult whole mount immunostained cerebella. The cerebella were sectioned serially in the transverse plane at 60 μm by

the vibratome (Microslicer DTK-2000; Dosaka EM Co). The sections were then treated with 3% H_2O_2 and 0.1% Triton X-100 in PBS for 30 min at room temperature to inactivate endogenous peroxidases. Following pre-incubation with PBS containing 10% normal goat serum and 0.1% Triton X-100 (blocking solution), the sections were reacted with anti-zebrin II (1:200) or anti-HSP25 (1:500), diluted in blocking solution, overnight at room temperature. After incubation, the sections were rinsed with PBS and incubated with a peroxidase-conjugated goat anti-mouse IgG (1:200; MBL) for zebrin II staining or a peroxidase-conjugated goat anti-rabbit IgG (1:200; MBL) for HSP25 staining. The immunoreactive products were visualized by incubating the tissue with 0.05% DAB and 0.015% H_2O_2 in PBS.

Results

Images of anti-zebrin II and anti-HSP25 immunostained whole mount cerebella processed using the original whole mount immunostaining protocol or our modified protocol are shown in Figure 1. Consistent with previous studies [5,6], whole mount immunostaining shows expression of zebrin II and HSP25 in Purkinje cells. A striking parasagittal array of zebrin II and HSP25 immunopositive Purkinje cell immunopositive stripes were observed using the original protocol (Fig. 1). Similar patterns of zebrin II and HSP25 stripes were also obtained using the modified heat-induced antigen retrieval protocol (Fig. 1). Heat treatment with an antigen retrieval reagent enhanced the staining intensity, especially for anti-HSP25 (Fig. 1). However, a slight increase in background staining was also observed (Fig. 1).

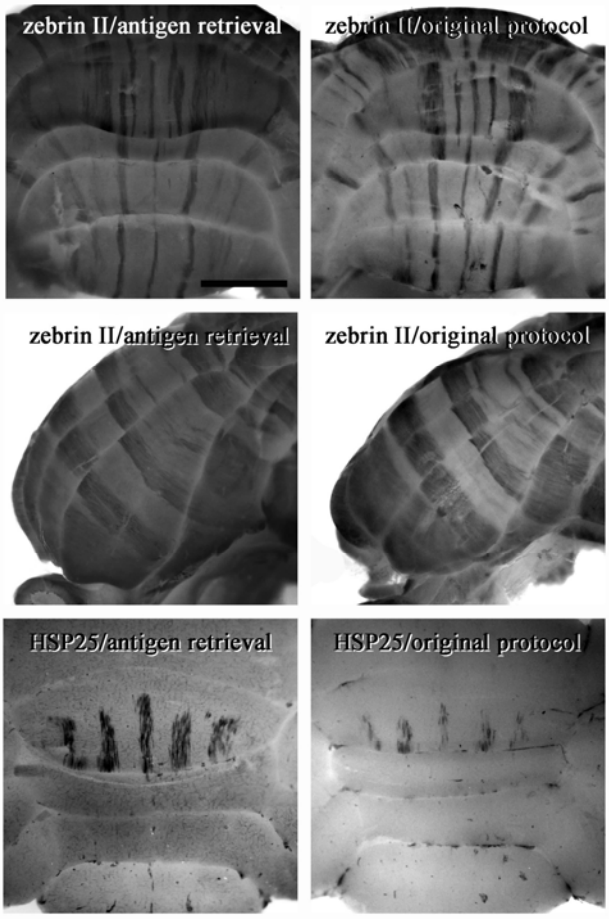

Figure 1. *Photographs of zebrin II and HSP25 whole mount immunostaining using the original protocol (right three photographs) and by the present modified protocol which includes heat induced antigen retrieval (left three photographs). Staining patterns of these two antigens were similar between the original and modified protocols. Staining intensity of HSP25 was somewhat enhanced by heat induced antigen retrieval. Scale Bar = 1 mm*

In order to determine the extent of antibody penetration, stained whole mount cerebella were sectioned and examined. A positive staining was observed in dendrites of particular subsets of Purkinje cells, which were organized into characteristic stripe patterns where zebrin II and HSP25 are known to be expressed (Fig. 2A, C). Distributions of these two antigens were identical to those obtained by the conventional section immunohistochemistry (Fig. 2B, D). Furthermore, a heat induced antigen retrieval procedure did not disrupt the cerebellar cytoarchitectures (Fig. 2A, C), indicating that the modified protocol maintains the integrity and morphology of cerebellar structures.

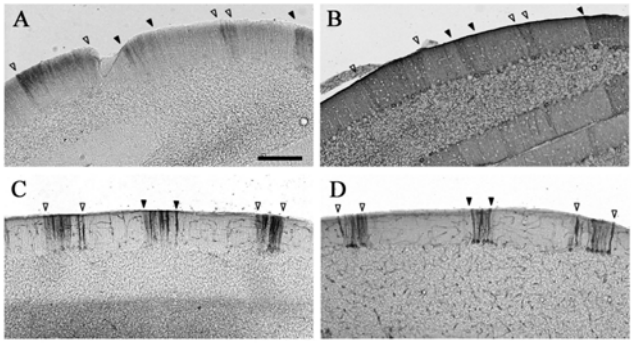

Figure 2. *Zebrin II and HSP25 immunostaining of trans transverse vibratome sections of whole mount zebrin II (A) or HSP25 (C) stained cerebella and conventional section immunohistochemistry for zebrin II (B) or HSP25 (D). Vibratome sections of whole mount cerebella showing zebrin II or HSP25 staining in dendrites of particular subsets of Purkinje cells. No disruption of cerebellar cytoarchitectures was observed. Distributions of these two antigens were identical to those obtained by conventional section immunohistochemistry. Bar = 20 μm*

Discussion

Antigen retrieval techniques such as delipidation with alcohols, microwave heating in buffers of different pH, and autoclaving, can greatly improve the immunohistochemical staining of formalin-fixed and paraffin embedded materials by recovering cryptic epitopes in the tissues [7]. The present study examined the application of heat induced antigen retrieval on whole mount immunestaining for the adult mouse cerebellum. The addition of this procedure circumvents the need to subject tissues to numerous freezing and thawing cycles while maintaining the integrity and morphology of cerebellar structures as observed by the staining patterns of two cerebellar compartmentation antigens, zebrin II and HSP25. Thus the heat induced antigen retrieval is applicable for whole mount immunostaining of adult mouse brains.

Sillitoe and Hawkes (2002) previously established a protocol for whole mount immunostaining using anti-zebrin II [5]. This protocol allows for visualization of the spatial organizations of other cerebellar compartmentation antigens [12] as well as particular components of the olivocerebellar tracts [6]. However, the most time consuming step in the original protocol is the tissue passage through 5 cycles of chilling to -80 ^{o}C and thawing to room temperature each for 60 min in 100% methanol before primary antibody addition. This step extends the protocol by 10 hr. The present study has successfully applied a heat induced antigen retrieval step to the whole mount immunostaining protocol which can substitute for the freeze/thaw cycles. Since the high temperature antigen retrieval procedure only takes 1 hr, this modified protocol allows for a more rapid and easy way to perform whole mount immunostaining of adult mouse brain tissues than the original protocol.

Whole mount immunostaining is a useful method for screening the spatial distributions of cerebellar compartmentation antigens in the adult mouse cerebella without the laborious task of sectioning and three dimensional (3D) reconstruction. This approach allows for examination of normal adult mouse cerebellar topography and patterning defects caused by mutations without 3D reconstruction of immunostained serial sections. The present modified protocol can contribute not only to screening the cerebellar topography and patterning defects but also to investigating other structures of the central nervous system, such as barrels in the primary somatosensory cortex and ocular dominance stripes and columns in the primary visual cortex.

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