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Review article

Whole-mount immunostaining protocol for screening of spatial organizations of cerebellar compartmentation

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Abstract

This paper introduces a protocol for whole-mount immunostaining adapted for the adult mouse cerebellum. This technique was originally described by Sillitoe and Hawkes (J Histochem Cytochem 50:235-244, 2002) for screening the parasagittal arrangements of cerebellar compartmentation antigens, i.e., zebrin II and heat shock protein (HSP25). We further optimized this technique to apply to heat-induced antigen retrieval, which provides a more rapid and efficient way to perform whole-mount immunostaining of adult tissues (Sawada and Sun, Curr Neurobiol 1:21-24, 2010). This approach allows for the visualization of spatial organizations of cerebellar examinations of the normal topography, particular components of olivocerebellar tracts, and patterning defects caused by mutations without 3D reconstruction of immunostained serial sections.

Key words: Immunohistochemistry, Zebrin II, HSP25, CRF, Ca^{2+} Channelopathy, Rolling mouse

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Introduction

Whole-mount immunostaining was developed on the basis of immunohistochemistry [1-3], and has become a widely used method to visualize the spatial distributions of specific antigens in embryos, fetuses, and dissected organs in whole mounts. Although the mild tissue processing procedure of whole-mount immunostaining allows for the improved preservation of antibodies in tissues, it is difficult to apply such procedures to dense and compact tissues, such as adult brain tissue, since the penetration of antibodies into tissues is limited to a depth of 8–9 μm [4].

In the cerebellum of mammalian species, the Purkinje cells form a complex arrangement of parasagittal stripes and transverse zones that is reflected in the diversity of gene expression patterns [5-8]. Currently, such parasagittal compartments are known to be formed by an alternating array of zebrin II-immunopositive and immunonegative Purkinje cell subsets [5]. The Purkinje cell compartments defined by zebrin II are closely related to projections of the particular components of olivocerebellar tracts [9-12]. However, an analysis of cerebellar compartmentation requires a 3D reconstruction of immunostained serial sections [13]. Sitolloe and Hawkes (2002) established a protocol for whole-mount immunostaining adapted to the adult mouse cerebellum using anti-zebrin II [14]. This protocol allows for the visualization of the spatial organizations of other cerebellar compartmentation antigens such as phospholipase Cβ3 and Cβ4 [15], patterned Purkinje cell degeneration caused by mutations [16], and for the projections of corticotrophin-releasing factor (CRF) immunopositive climbing fiber projections [12]. This paper introduces a protocol for whole-mount immunostaining, which is optimized to visualize the spatial organizations of the cerebellar compartmentation antigens, and for our studies in the detection of patterning defects of the cerebellar compartmentation in an ataxic mutant, rolling mouse Nagoya.

Original protocol

A protocol of whole-mount immunostaining adapted for the adult mouse cerebellum by Sitolloe and Hawkes (2002) is shown as follows [14]. The cerebella, which are perfused and postfixed with 4% paraformaldehyde, are usually used for this approach. Ethanol can be substituted for methanol, which is contained in Dent's fixative and is used for dehydration in the following protocol [17].

Step 1: Post-fix in Dent's fixative (methanol: dimethylsulfoxide (DMSO) = 4:1) overnight at room temperature (RT).

Step 2: Immerse in Dent's bleach (methanol:DMSO: 30% H_2O_2 = 4:1:1) overnight at RT to inactivate endogenous peroxidase.

Step 3: Wash tissues 3 times with 100% ethanol for 60 min each.

Step 4: Subject tissues to 5 cycles of chilling to -80 ^oC and thawing at RT in 100% methanol.

Step 5: Rehydrate tissues for 90 min each with 50% methanol, 15% methanol and PBS.

Step 6: Enzymatically digest tissues 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 tissues three times with PBS for 2 hr each.

Step 8: Incubate tissues with PBS containing 2% non-fat skim milk and 0.1% Triton X-100 (PBSMT) overnight at 4 ^oC.

Step 9: Incubate tissues for 48 hr at 4 ^oC with the primary antibody in PBS containing 10% normal goat serum (NGS), 0.1% Triton X-100 and 5% DMSO.

Step 10: Rinse tissues with PBSMT for 10 min each.

Step 11: Incubate tissues for 24 hr at 4 ^oC with a peroxidase-conjugated secondary antibody in PBSMT containing 5% DMSO.

Step 12: Wash tissues twice with PBSMT for 2 hr at 4 ^oC.

Step 13: Rinse tissues with PBS containing 0.2% bovine serum albumin and 0.1% Triton X-100 (PBT) for 2 hr at 4 ^oC.

Step 14: Incubate tissues 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

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 [18]. We modified the original protocol by applying heat-induced antigen retrieval, and succeeded in devising a more rapid and efficient way to perform whole-mount immunostaining [17;19]. The heat-induced antigen retrieval procedure is described as follows. The following procedure was performed in substitution for *Steps 3-6* of the original protocol.

A) Rehydrate tissues with 50% methanol, 15% methanol and PBS for 90 min each.

B) Treat tissues with Antigen Retrieval Reagent UNIVERSAL (R&D system, Minneapolis, MN, lot #950512) for 30 min in a 90 ^oC water bath.

C) Cool tissues for 30 min at 4 ^oC.

Heat-induced antigen retrieval allows the penetration of antibodies throughout the cerebellar cortex with no disruption of the cerebellar cytoarchitectures [17;19]. Vibratome sections of zebrin II-immuostained whole cerebella showed positive staining in the dendrites of particular subsets of Purkinje cells, the distribution of which was identical to that obtained by conventional section immunohistochemistry (Fig. 1). In the original protocol, the cerebella is passed through 5 cycles of chilling to -80 ^oC followed by thawing at room temperature for 60 min each in 100% methanol prior to the primary antibody addition to improve the penetration of the antigens into tissues [14]. That step extends the protocol by 10 hr, whereas a heat-induced antigen retrieval step can substitute for such freeze/thaw cycles in the original protocol of whole-mount immunostaining [17]. Since a heat-induced antigen retrieval procedure takes only 1 hr, this procedure allows for a simpler, more rapid and easy way to perform whole mount immunostaining of adult mouse brain tissues than that in the original protocol.

Visualization of spatial organization of cerebellar compartmentation antigens

Figure 2 shows whole-mount cerebella immunostained for zebrin II and heat shock protein 25 (HSP25) in the adult mice. Four transverse expression domains ("zones") in the cerebellum have been defined based on the expression pattern of zebrin II: the anterior zone (AZ: lobules I–V) – stripe; the central zone (CZ; lobules VI to VII) – uniform; the posterior zone (PZ; lobules VIII to dorsal IX) – stripe; and the nodular zone (NZ; ventral lobule IX to lobule X) – uniform (Fig. 2) [8;13;14]. Such a pattern of zebrin II expression is known to alternate complemental to phospholipase Cß4 [15]. Whereas zebrin II expression uniformly appeared in the CZ and NZ, these two both further subdivided into a reproducible array of parasagittal stripes defined by the expression patterns of the HSP25 (Fig. 2) [8] and human natural killer cell antigen 1 (HNK 1) [20;21]. Such Purkinje cell patterns are central to normal cerebellar organization [14], and their disruption triggers severe motor control problems [22-24].

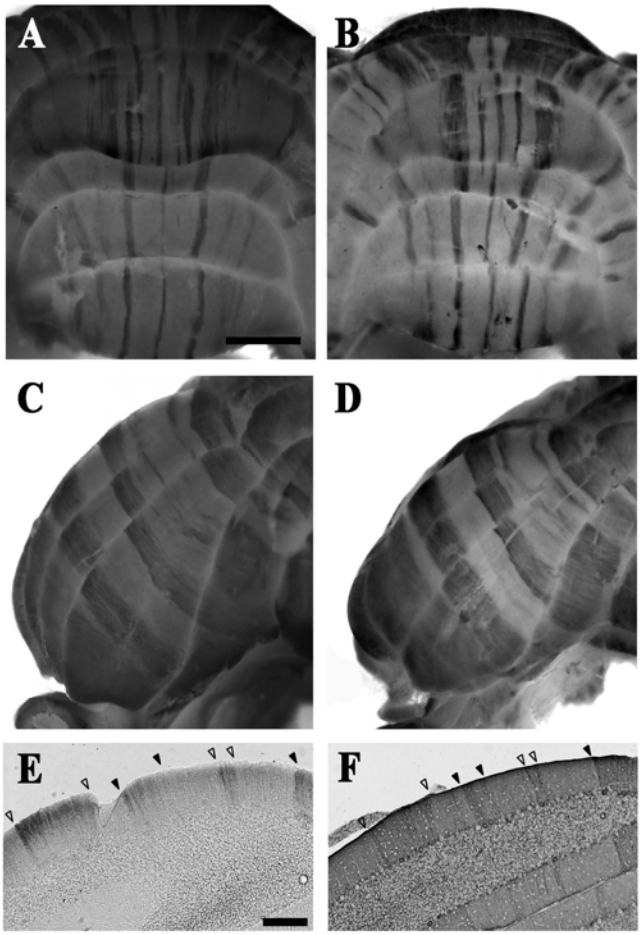

Figure 1. ***Photographs of whole mount zebrin II immunostaining by using the present modified protocol which includes heat induced antigen retrieval (A,C) and the original protocol (B, D) and, and zebrin II immunostaining of transverse vibratome sections of whole-mount zebrin II stained cerebella (E) and transverse vibratome sections by conventional section immunohistochemistry (F).*** *Staining patterns of zebrin II were similar between the original and modified protocols (A-D). Vibratome sections of whole mount cerebella showing zebrin II staining in dendrites of particular subsets of Purkinje cells with no disruption of cerebellar cytoarchitectures. Distribution of zebrin II-immunopositive Purkinje cells were identical to that obtained by conventional section immunohistochemistry. Bar = 1 mm in (A) [Applied to (B), (C) and (D)]. Bar = 20 μm in (E) [Applied to (F)] (Adapted from Ref. 17).*

Visualization of particular components of olivocerebellar tracts

Though CRF is a 41-amino acid peptide that is primarily thought of as a stress-related hypophysiotropic hormone,

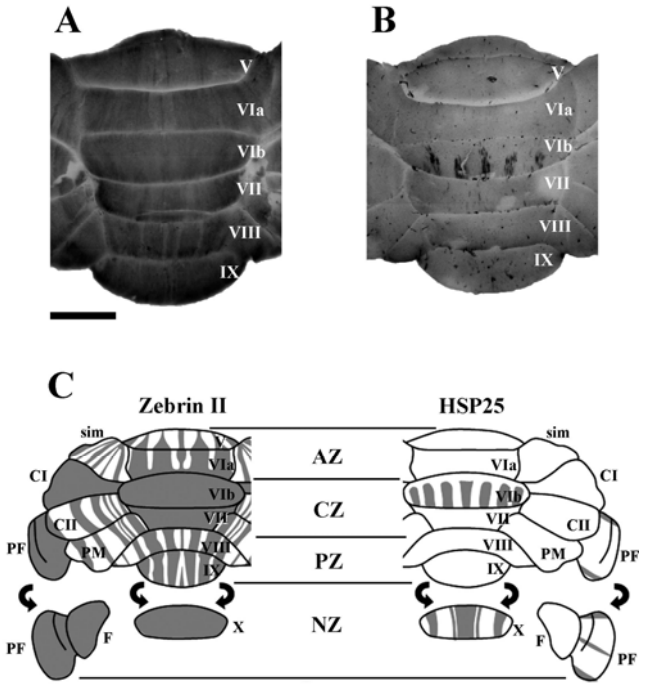

Figure 2. Cerebellar compartments defined by zebrin II and HSP25 immunostaining in the mouse. *A: Dorsal view of zebrin II-stained whole-mount cerebellum. B: Dorsal view of HSP25-stained whole-mount cerebellum. C: Summary of the topographical distribution of zebrin II in the mouse cerebellum. D. Summary of the topographical distribution of HSP25. Four transverse expression domains ("zones") in the cerebellum have been revealed in the cerebellum based on the expression pattern of zebrin II: the anterior zone (AZ: lobules I–V) – stripe; the central zone (CZ; lobules VI to VII) – uniform; the posterior zone (PZ; lobules VIII to dorsal IX) – stripe; and the nodular zone (NZ; ventral lobule IX to lobule X) – uniform. Whereas zebrin II is uniformly expressed in the CZ and NZ, those two zones are further subdivided into a reproducible array of parasagittal stripes defined by the expression patterns of the HSP25. CI: crus I of ansiform lobule; CII: crus II ansiform lobule; F: flocculus; PF: paraflocculus; sim: lobulus simplex. Bar = 1 mm (From Ref. 19).*

it is also widely implicated as a neurotransmitter throughout the central nervous system. In the cerebellum, CRF is present in the subsets of climbing and mossy fibers, serving as a neuromodulator that enhances the glutamate sensitivity of Purkinje cells [25-27] and the induction of a long-term depression at the climbing fiber-Purkinje cell synapse [28]. Interestingly, CRF-immunopositive climbing fibers form a striking parasagittal pattern in the cerebellum, which resembles the expressionpattern of zebrin II in several mammalian species such as non-human primates [29], cat [30], opossum [31],

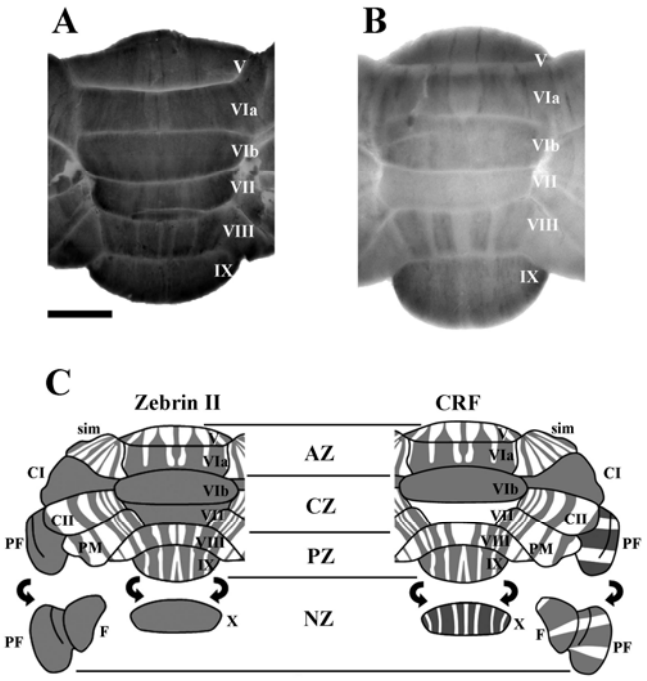

Figure 3. Photographs of zebrin II (A) and CRF (B) stained whole-mount cerebella, and summaries of the topographical distributions of zebrin II and CRF in the mouse cerebellum (C). *Whole-mount staining patterns are similar, but do not completely overlap between zebrin II and CRF. Note the absence of CRF immunostaining from lobule VII and the presence of only four CRF-immunopositive stripes in lobule VIII. CI: crus I of ansiform lobule; CII: crus II ansiform lobule; F: flocculus; PF: paraflocculus; sim: lobulus simplex. Bar = 1 mm (adapted from Ref. 12).*

rabbit [32], and mice [33]. In our previous study, whole-mount immunostaining revealed that CRF-immuno positive climbing fiber projections did not completely overlapp with the distribution of zebrin II-immuno- positive Purkinje cells (Fig. 3) [12]. The parasagittal array of CRF-immunopositive stripes in the AZ and the hemispherical regions of the CZ and PZ was generally similar to that revealed by zebrin II expression (Fig. 3). However, in the vermis of the CZ, PZ and NZ, the pattern of CRF-immunopositive climbing fiber projections did not precisely match the pattern of zebrin II expression. In the CZ, CRF-immunopositive climbing fiber terminals were plentiful in lobule VIb but absent from lobule VII, whereas zebrin II was expressed uniformly (Fig. 3).

In lobule VIII (the rostral PZ), the array of CRF-immunopositive climbing fiber stripes did not coincide with those of the zebrin II-immunopositive Purkinje cell stripes, while CRF-immunopositive stripe 2 shared its lateral boundary with the medial edge of P4+ but its medial boundary split P3+ in half (Fig. 3). CRF-immunopositive climbing fiber stripes were also present in lobules IX and X, the flocculus and paraflocculus (NZ), whereas zebrin II immunostaining was expressed uniformly in those regions (Fig. 3).

Some CRF-immunopositive climbing fibers were distributed, corresponding to HSP25-immunopositive Purkinje cell stripes in the NZ (Fig. 3).

Visualization of patterning defects in the cerebellum

Patterning defects in the cerebellum are revealed in mutant mice by whole mount immunostaining, e.g., a patterned Purkinje cell loss in whole-mount cerebella immunostained for Calbindin D-28 k in lurcher and Niemann-Pick type mice [14,16]. We here introduce our recent study that revealed a striking pattern of parasagittal stripes of ectopic tyrosine hydroxylase (TH) expression in the cerebellum of rolling mice by whole mount immunostaining [34].

Rolling mice are an ataxic mutant mouse first described by Oda (1973) [35] and characterized by a severe ataxic gait and abnormal hindlimb extension [36;37]. This mutant mouse carries a mutation in a recessive autosomal allele of the tottering locus on chromosome 8 [35] that encodes a gene for the α_{1A} subunit of P/Q-type Ca^{2+} channel ($Ca_V2.1$) [38], as do tottering, leaner [39], rocker [40] and wobbly [41] mice. In humans, defects in this gene are responsible for several neurological disorders such as episodic ataxia type-2 (EA-2) and familial hemiplegic migraine [42]. We previously reported an ectopic expression of TH in particular subsets of Purkinje cells, which were similar to the expression pattern of zebrin II, in the cerebellum of rolling mice [43-47]. Such an ectopic TH expression is thought to reflect an increase in the intracellular Ca^{2+} concentration of the Purkinje cells [44]. However, ectopic TH expression in the Purkinje cells was not specific to the $Ca_v2.1$ mutants, since it has been observed in other allelic mutant mice such as dilute-lethal [43;48] and Neimann-Pick type C1 [49].

Whole-mount immunostaining revealed such an ectopic TH expression in the cerebellum of rolling mice with a zebrin II-like expression pattern (Fig. 4). However, expression patterns between TH and zebrin II did not completely overlap. Whereas zebrin II was uniformly expressed throughout the CZ (lobules VI to VII) and NZ (ventral IX and lobule X), TH-immunopositive Purkinje cell stripes were present in those regions with an alternating array toward HSP25-immunopositive Purkinje cell stripes (Fig. 4) [34]. Section-double immunohisto chemistry revealed that some but not all TH-immunopositive

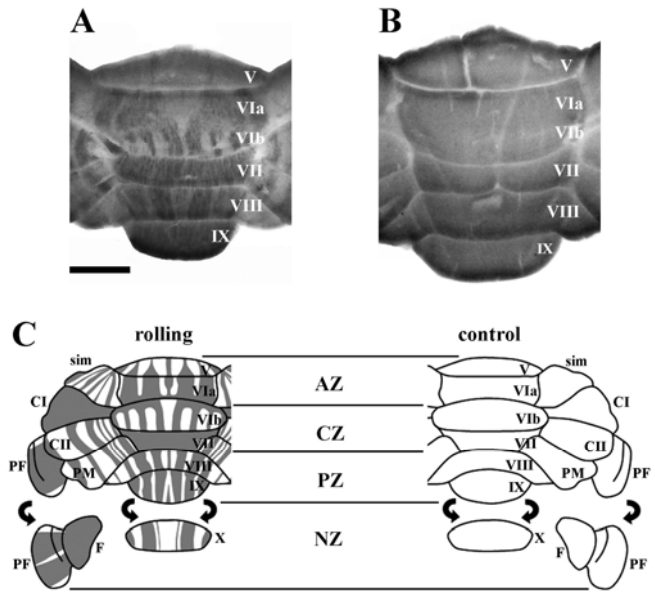

Figure 4*. Photographs of TH stained whole-mount cerebella in rolling (A) and wild-type (B) mice, and summaries of the topographical distribution of TH in these two mice (C)**. Five narrow TH-immunopositive stripes were observed in lobules I to V. An array of TH-immunopositive stripes was also obvious more laterally in the lobulus simplex. Although TH-immunopositive Purkinje cell subsets formed symmetrical stripes anteriorly in lobules V–VIb, and more posteriorly in lobules VIII–IX, these cell subsets delineated a TH-immunopositive transverse zone of lobule VII. TH-immunopositive stripes are evident in lobule X, and the paraflocculus. CI: crus I of ansiform lobule; CII: crus II ansiform lobule; F: flocculus; PF: paraflocculus; sim: lobulus simplex. Bar = 1 mm (From Ref. 19).*

stripes shared common boundaries with HSP25- immunopositive stripes in those regions [34].

These results suggest that the constitutive expression of HSP25 prevents the ectopic expression of TH in zebrin II-immunopositive Purkinje cell subsets in the cerebellum of rolling mice.

Conclusions

Whole-mount immunostaining is a widely used method for visualizing the spatial distributions of specific antigens in embryos, fetuses, and dissected organs in a whole mount. Optimization of this technique for the adult mouse cerebella serves to clarify the spatial distributions of cerebellar compartmentation antigens (i.e., zebrin II and HSP25) [14,15], particular subsets of climbing fibers [12], urkinje cell loss in mutant mice [14;16], and the ectopic expression of the specific antigens [34;46;47] without the laborious task of sectioning and 3D reconstruction. This approach allows us not only to screen out the cerebellar topography and patterning defects but also to investigate other structures of the 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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