

Post-diaminobenzidine Treatments for Double Stainings: Extension of Sulfide-Silver-Gold Intensification for Light and Fluorescent Microscopy

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Summary

Double staining protocols using the most popular immunoperoxidase techniques may raise difficulties. The two ordinary detection systems may cross-talk, when the primary antibodies are derived from phylogenetically closely related animals. A color shift of the 3,3'-diaminobenzidine (DAB) polymer may occur during the second development, resulting in poor distinction between the two kinds of deposits. A post-DAB technique, sulfide-silver-gold intensification, was fine tuned to eliminate these difficulties, which may be especially suitable for colocalization of cell nuclei and perikarya of the same cells. The revised method was probed in combination with a subsequent other immunoperoxidase step or fluorochrome-tagged reagents. The nuclear antigens (BrdU, c-Fos, and Prox-1) were first visualized with DAB polymer, which were then treated with SSGI, turning the deposit black. Thereafter, cytoplasmic antigens (doublecortin, neuronal nuclei, and calbindin) were detected with either another immunoperoxidase using DAB again or immunofluorescence labeling. In both approaches, the immunopositive nuclei and cytoplasmic sites could be easily distinguished even at low magnifications. Different shielding or eluting posttreatments were compared for consecutive acetylcholinesterase histochemistry terminated with DAB development and immunohistochemistry in the same sections. In conclusion, we recommend post-DAB treatments that abolish interactions between detection systems and allow clear distinction between the two signals under various conditions. (J Histochem Cytochem 68: 571–582, 2020)

Keywords

acetylcholinesterase histochemistry, DAB, diisopropyl fluorophosphate, double staining, immunofluorescence, immunohistochemistry, silver enhancement

Introduction

3,3'-diaminobenzidine (DAB) is the most frequently used chromogen in horseradish peroxidase (HRP)-based immunohistochemistry and neuroanatomic tract-tracing experiments.¹ The enzyme-catalyzed oxidation of DAB by hydrogen peroxide results in a brown polymer deposit, the color of which can be changed to bluish black in the presence of nickel (Ni) ions² or cobalt ions,³ permitting double staining by means of DAB at different ionic compositions.⁴

Nevertheless, the water-insoluble end product can spatially hinder proper subsequent detection of the antigens during the double immunolabeling procedure. Therefore, immunofluorescence detections are preferred for multiple

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labeling. However, if the two targeted antigens are located in different cells or in separate cellular compartments of the identical cell, HRP-based techniques can also be taken into consideration. The consequent applications of HRP techniques in double stainings require at least the following conditions to be fulfilled: (1) the immunoreagents of the two detection systems will not interfere with each other, (2) the two target molecules will be located in spatially separate tissue or cell compartments, (3) the end products will be stable throughout the colocalization procedure, (4) the deposits should be clearly distinguished by their colors, and (5) the immunohistochemical noise should be kept low, otherwise the immunoreagents will not have access to marker molecules.

These requirements can be achieved by silver intensification techniques,^{5–7} which are usually not considered to be the methods of choice for double labeling. This may be due to (1) some technical difficulties including the demand for extra clean bench work, which exceeds the need of the routine histochemistry; (2) the general belief related to the word “intensification” that these techniques are to be used for only signal amplification purposes; and (3) mystery, which the silver ion-based techniques are wrapped in, regarding their inflexibility to various conditions. In this report, we would like to dispel these concerns by shifting the emphasis of our sulfide-silver-gold intensification (SSGI) method (Dobo, 2011) from its intensification power to versatility and reproducibility.

Silver intensification techniques use the catalytic property of the DAB polymer to reduce silver ions to fine metal grains by formaldehyde⁸ or ascorbic acid.⁹ The atomic silver is then usually substituted with gold in either gold(III) chloride⁵ or gold(I) rhodanide.⁷ Despite the well-documented advantages of these silver-based post-DAB treatments, their improper uses may enhance inherent tissue argyrophilia, which may intensify the noise of immunohistochemistry, impairing the success of the subsequent staining. We were prompted to dispel possible and often justified aversion to applications of post-DAB treatments by simplification and flexible, fine adjustment of the working solutions for balanced and reproducible double staining. Although these post-DAB techniques are collectively referred to as intensification ones, we lay stress on their other benefits, such as their capacity for color conversion and shielding the first immunolabeling.

The latter feature was thought to capacitate the silver-based post-DAB treatments for adaptation of the HRP-based immunohistochemistry to colocalization with immunofluorescence, both of which approaches have their own advantages, drawbacks, and technical difficulties. In double staining, a common crucial difficulty is that either the primary antisera should be

derived from different species or expensive, affinity-purified secondary antibodies raised to highly specific epitopes of different IgG subclasses have to be used. It was conceived to eliminate the restriction of the application of cross-talking antibodies by means of masking the first one with a silver-based post-DAB treatment. Our SSGI⁷ was especially adapted for double labeling with HRP-based immunohistochemistry and a consecutive another immunoperoxidase or immunofluorescence technique. This novel combination was envisaged to be advantageous for the colocalization of the smaller nuclei containing one of the target antigens, terminated and shielded with the SSGI method, and the larger perikarya having the other marker to obtain optimal visual distinction.

Various post-DAB treatments were probed in the cases where one (immuno)histochemical substance is ample. For instance, acetylcholinesterase (AChE) is abundant in cholinceptive structures. AChE enzyme histochemistry is one of the most often used methods in the histological examination of brain. This method has been well exploited to produce precise maps of brain areas of various mammals,^{10–15} which are required references in modern neuroanatomy. The modern, improved AChE histochemistry includes DAB development due to the HRP-like catalytic property of its primary reaction end product.¹⁶ Despite its easy use, it was found to be difficult to apply this technique in colocalization with immunohistochemistry. In our study, we recommend easy, reproducible solutions for double labeling of AChE by means of enzyme histochemistry and other neuronal markers by immunohistochemistry in combination with some post-DAB treatments without compromise.

Materials and Methods

Animals

Adult male Wistar rats (150–200 g) and adult male NMRI mice (25–30 g) were kept in a temperature-controlled room under standard light/dark cycle, with food and water ad libitum. All experimental procedures were conducted according to the EU Directive (2010/63/EU) and the Hungarian Animal Act. Specific approval of care and use of animals was obtained in advance from the Faculty Ethical Committee on Animal Experiments (University of Szeged).

Treatments

For labeling the proliferating cells of the hippocampal dentate gyrus, 5-bromo-2'-deoxyuridine (BrdU) was

repeatedly injected into mice on five consecutive days (100 mg/kg, IP; Sigma-Aldrich, St. Louis, MO). c-Fos expression was enhanced in rats by 4-amino-pyridine (4-AP)-induced seizures observed 20 min after the injection (5 mg/kg, IP; Sigma-Aldrich). To irreversibly block AChE activity in the processes and to evoke de novo AChE synthesis in the perikarya, diisopropyl fluorophosphate (DFP) (10 mg/kg, IM; Sigma-Aldrich) was used in mice.¹⁷ The vegetative effects of the AChE inhibitor were counterbalanced with atropine injection (5 mg/kg, IP; Sigma-Aldrich) before DFP administration. The DFP-treated animals were sacrificed after a 6-hr survival period.

Tissue Preparation

BrdU-treated mice were sacrificed 2 weeks after the injections. Rats with fully developed tonic-clonic convulsions were sacrificed 90 min after 4-AP injections. The other animals were deeply anesthetized with diethyl ether and perfused through the ascending aorta with physiological saline and then with 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were dissected and cryoprotected overnight in buffered 30% sucrose at 4°C. Coronal brain sections were cut on a freezing microtome at a thickness of 24 µm.

Immunohistochemistry

The free-floating sections were treated with 0.5% Triton X-100 and 3% hydrogen peroxide, and then with normal swine serum (dilution: 1/10). The following primary antibodies were used: rat anti-BrdU (dilution: 1/400; Harlan Sera-Lab, Loughborough, UK); rabbit anti-calbindin (CALB; dilution: 1/2000; Chemicon, Temecula, CA); goat anti-calretinin (CALR; dilution: 1/4000; Millipore, Temecula, CA); goat anti-choline acetyltransferase (ChAT; dilution: 1/100; Millipore); goat anti-doublecortin (DCX; dilution: 1/500; Santa Cruz Biotechnology, Dallas, TX); rabbit anti-c-Fos (dilution: 1/2000; Santa Cruz Biotechnology); rabbit anti-neuronal nuclei (NeuN; dilution: 1/8000; Chemicon); and rabbit anti-Prox-1 (dilution: 1/12,000; Chemicon).

The sections were incubated under continuous agitation at room temperature overnight. After washing, the sections were incubated with the appropriate biotinylated secondary antibody (dilution: 1/400; Jackson ImmunoResearch, West Grove, PA) for 60 min and finally with HRP-labeled streptavidin (dilution: 1/1000; Jackson ImmunoResearch) for 60 min. The sites of immunoreaction were visualized with DAB in the absence or presence of Ni.²

Silver-intensified Gold Labeling

The DAB-Ni developed sections were further processed by SSGI published earlier,⁷ which was specifically modified for light microscopic investigation as follows. The specimens were first treated with a sulfide solution (0.023 M Na₂S·9H₂O in 0.2 M sodium phosphate buffer, pH 7.4) for 5 min. The sections were then rinsed with 2% sodium acetate four times. After the third wash, the sections were transferred into glass vials thoroughly cleaned with Farmer's solution.¹⁸

The optimal silver intensification requires proper adjustment of the volumetric proportion of the following components—A: 0.2% silver acetate, B: 0.5% hydroquinone in 2 M citrate buffer, C: 2% sodium acetate, and D: 2 M citrate buffer (pH 3.5–4) composed of 1.2 M citric acid monohydrate and 0.8 M trisodium citrate dihydrate. To receive mild color conversion, an equal volumetric proportion of the four components were used (A:B:C:D = 1:1:1:1). For a significantly stronger signal, the proportion of the components was modified omitting component D (A:B:C = 1:2:1), whereas the highest signal-to-noise ratio was achieved by mixing the two major components (A:B = 1:1). In each mixture, component A was added last and dropwise to the hydroquinone-containing solution. In either version of development, the sections were treated for 7 to 10 min to get the best results. Following the silver treatment, the sections were quickly rinsed with 2% sodium acetate.

The samples were then treated with gold(I) rhodanide for 10 min, which was freshly prepared as follows: 0.2 ml of 2% HAuCl₄ was added dropwise and slowly to 8 ml of 1.5% potassium thiocyanate (KSCN) under constant and intensive vortex mixing until the temporary red color disappears within approximately 5 min.⁷ Following this gold toning, the sections were quickly rinsed once with 2% sodium acetate. The ionic gold was removed from the tissue with 3% Na₂S₂O₃·5H₂O for 5 min. Finally, the sections were rinsed three times with 2% sodium acetate.

Immunofluorescence and Confocal Microscopy

Sections were incubated with Alexa Fluor 488–, Alexa Fluor 594– and Cy3-conjugated secondary antibodies or streptavidin (dilution: 1/200; Jackson ImmunoResearch). The fluorochrome-labeled samples were mounted onto glass slides with Depex. The fluorescence images of the immunostained samples were captured using an Olympus Fluoview (Fv10i; Olympus, Tokyo, Japan) confocal laser scanning microscope. The stained specimens were prescanned with the selected fluorescence filter, and then the objects of

Table 1. Double Immunohistochemistry for Light and Combined Bright-field/Fluorescence Microscopy Including Antibodies to the Cell Markers, Labels (DAB With/Without Ni, Post-DAB Treatment, Fluorochrome), and Referred Figures.

Double Immunohistochemistry	First Antibody	First Label + Post-DAB	Second Antibody	Second Label	Figures
BrdU–DCX	Rat anti-BrdU	DAB-Ni + SSGI	Goat anti-DCX	DAB	1A
Prox-1–CALB	Rabbit anti-Prox-1	DAB-Ni + SSGI	Rabbit anti-CALB	DAB	1B
c-Fos–NeuN	Rabbit anti-c-Fos	DAB-Ni + SSGI	Rabbit anti-NeuN	DAB	1C
BrdU–DCX	Rat anti-BrdU	DAB-Ni + SSGI	Goat anti-DCX	Cy3	2A–C
Prox-1–CALB	Rabbit anti-Prox-1	DAB-Ni + SSGI	Rabbit anti-CALB	Cy3	2D–F
c-Fos–NeuN	Rabbit anti-c-Fos	DAB-Ni + SSGI	Rabbit anti-NeuN	Cy3	2G–I

Abbreviations: DAB, diaminobenzidine; BrdU, 5-bromo-2'-deoxyuridine; DCX, doublecortin; CALB, calbindin; c-Fos, FBJ murine osteosarcoma cellular oncogene; NeuN, neuronal nuclei; Prox-1, Prospero Homeobox 1; SSGI, sulfide-silver-gold intensification.

interest were marked and examined with 60× water immersion objective lens.

Double Immunostaining

The nuclear antigens were visualized first with primary antibodies, and the immunoreactions were revealed with the bluish-black DAB-Ni. The signal was stabilized with silver-intensified gold labeling, which allows the subsequent application of either the HRP-based or the immunofluorescence technique. For the light microscopic observation of double immunostaining, perikaryonal antigens were stained with brown DAB. For the combined light fluorescent microscopy, the sections were labeled for the perikaryonal antigen with fluorochrome-tagged immunoreagents (Table 1).

Combination of Immunohistochemistry and AChE Enzyme Histochemistry

If the immunohistochemistry was applied first, it was performed as described earlier and completed using DAB without Ni (version 1) or with Ni (version 2). Afterward, the sections were treated with 0.5% Triton X-100 and 3% hydrogen peroxide for 10 min to inactivate HRP before AChE staining. The detection of AChE was accomplished as follows. The nonspecific esterase activity was blocked with ethopropazine HCl (0.2 mM; Sigma-Aldrich) for 30 min. The incubation medium was obtained by a 1:40 dilution of Karnovsky's solution composed of 7.5 ml of 0.1 M maleate (pH 6), 0.5 ml of sodium citrate, 1 ml of 30 mM CuSO₄, 0.8 mL of 5 mM K₃[Fe(CN)₆], and 5 mg of acetylthiocholine iodide.¹⁶ Enzyme histochemistry was performed for 20 to 30 min. The reaction end product having HRP-like activity was visualized in version 1 by the bluish-black DAB-Ni polymer and in version 2 by the brown DAB polymer.

In the transposed sequence of double staining, the AChE staining was terminated by means of DAB in the

presence of Ni (version 3) or absence of Ni (version 4). In version 3, the sections were treated with sodium sulfide and subsequently gold-toned with freshly prepared more concentrated gold(I) rhodanide (0.3 mL of 2% HAuCl₄ was added dropwise and slowly to 4 mL of 1.5% KSCN, similarly as described above). By these steps, bluish black was converted to shades of brown. In version 4, the stained samples were treated with 1% sodium EDTA (pH 7.0) for 30 min to dissolve the primary end product (presumably Hatchett's brown, that is, Cu₂[Fe(CN)₆]), which would otherwise interfere with the subsequent immunoperoxidase staining, preserving the brownish color of the DAB polymer. In both version 3 and version 4, the use of silver-containing solution was omitted, and the subsequent immunoreactions were visualized with the bluish-black DAB-Ni polymer.

The sections stained in version 3 were obtained from untreated mice, whereas the sections stained in the other three versions of the combination of immunohistochemistry and AChE enzyme histochemistry were derived from DFP-injected mice (Table 2).

Light Microscopy Image Processing

Pictures were taken with an image capture system (Olympus DP50) attached to an Olympus BX-50 microscope with 60× objective lens. (Soft Imaging System GmbH; Münster, Germany). The images were optimized for best contrast and brightness with Adobe Photoshop program 7.0, but only those functions that treated all pixels in the images equally were used.

Results

Single Immunostaining

Immunoreactivities for nuclear antigens,^{19–21} such as BrdU, c-Fos, Prox-1, and neuronal perikaryonal antigens,^{22–24} such as DCX, NeuN, and CALB, were found

Table 2. Combinations of Immunohistochemistry and AChE Enzyme Histochemistry Including Animal Pretreatment, Neuronal Markers, Labels (DAB With/Without Ni), Post-DAB Treatments, and Referred Figures.

Versions	Pretreatment	First Marker	First Label	Post-DAB	Second Marker	Second Label	Figures
Version 1	DFP	ChAT	DAB	H ₂ O ₂	AChE	DAB-Ni	3A
Version 2	DFP	ChAT	DAB-Ni	H ₂ O ₂	AChE	DAB	–
Version 3	None	AChE	DAB-Ni	Na ₂ S + AuSCN	CALR	DAB-Ni	3B
Version 4	DFP	AChE	DAB	EDTA	ChAT	DAB-Ni	3C

Abbreviations: AChE, acetylcholinesterase; DAB, diaminobenzidine; CALR, calretinin; DFP, diisopropyl fluorophosphate; ChAT, choline acetyltransferase; HRP, horseradish peroxidase.

in those neuronal elements by means of our single stainings, which were already published by others. The results of our single immunostainings are beyond the scope of this study; therefore, these are not shown here, but described briefly as follows.

The sites of BrdU uptake were immunolabeled in small nuclei, which were either scattered in the brain or alternatively were accumulated under the ependymal cells of the lateral ventricles and subgranular zone of the dentate gyrus. c-Fos immunostaining, greatly enhanced with 4-AP pretreatment, was found in a large number of nuclei throughout the brain. High density of immunoreactivity for Prox-1 was observed in many brain areas, among which the granule cell layer of the dentate gyrus was the most prominent.

DCX-like immunoreactive somata and extensive dendritic trees of the granule cells were observed in the dentate gyrus. Immunoreactivity for NeuN was found in almost all neurons of the rodents, which appeared not only in the perikarya and their proximal dendrites but in their cell nuclei as well. CALB immunostaining was detected in a great number of multipolar neurons throughout the brain.

Double Immunostaining for Light Microscopic Observation

Antigens present in different cell compartments of the same cell were stained with the same peroxidase-based technique covering the first stained antigens with SSGI, as shown in Fig. 1. These nuclear antigens (BrdU, Prox-1, and c-Fos) were labeled with the bluish-black Ni-enhanced DAB that was followed by sulfide treatment, silver deposition, and its replacement with gold. The treatment with gold(I) rhodanide strengthened the color intensity of the nuclei without altering their black appearance during the further incubation steps. The elementary gold successfully blocked the overstaining of the antigen–antibody complexes in the consecutive detection of the second signal.

The presence of the first reaction end product in the nuclei did not interfere with the appearance of the

subsequently stained cytoplasmic antigens (DCX, CALB, and NeuN). The fine processes of neurons were visualized by the brown DAB polymer.

The combinations of antibodies applied in the current work are listed in Table 1. It is important to note that this double immunolabeling protocol made it possible to apply two kinds of antibodies raised in the same species (e.g., rabbit anti-c-Fos and rabbit anti-NeuN, rabbit anti-Prox-1 and rabbit anti-CALB) in colocalization without using expensive subtypes of the primary antibodies.

The Combination of SSGI With Immunofluorescence

Images of double-labeled brain tissue captured by confocal laser scanning microscopy are shown in Fig. 2. The application of immunofluorescence for DCX, CALB, and NeuN on the gold-toned sections resulted in bright labeling in the cytoplasm (Fig. 2A–C) comparable with the immunoperoxidase-stained sections. In fractions of the immunolabeled perikarya, the cytoplasm enclosed dark roundish areas, which turned to be gold-containing nuclei when viewed in corresponding phase-contrast images (not shown). Overlaying of the fluorescent and phase-contrast images confirmed the double labeling of BrdU, Prox-1, or c-Fos-immunopositive nuclei and the neuronal perikarya (Fig. 2D–F). The first immunohistochemical labeling producing the blackish nuclear reaction end product did not interfere with the fluorescent visualization of the second cytoplasmic antigen even in those cases when both primary antibodies were produced in the same animal species. Interestingly, when permanent mounting medium Depex was used, instead of aqueous medium like Vectashield, the Cy3-conjugated immunoreagents seemed to be the most effective in terms of longevity and intensity out of the tested fluorochromes in our hands.

It is worth noting that the DAB and SSGI slightly increased the general fluorescent background, but a proper adjustment of the threshold values in laser

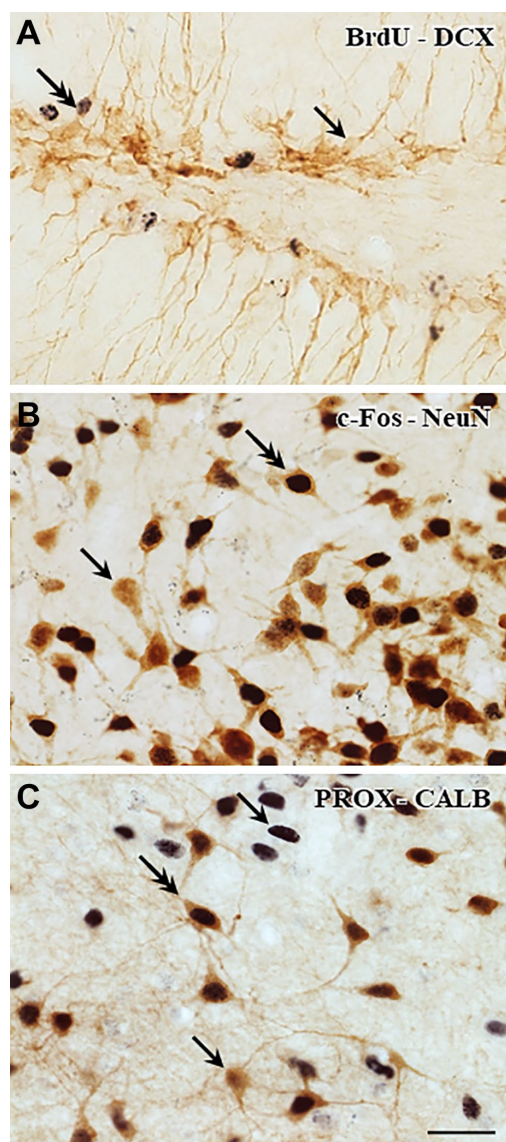


Figure 1. Double immunohistochemical staining for light microscopy. Nuclear antigens were stained with primary antibodies against BrdU, Prox-1, and c-Fos, which were revealed with DAB-Ni. Immunostaining was followed by SSGI, resulting in black color. Subsequently, perikaryonal staining (DCX, CALB, and NeuN) was carried out applying the same peroxidase-based technique, and it was visualized with brown DAB. Single-head arrows point to single-labeled structures, and the arrows with double heads mark the neurons with immunopositivity both in their nuclei and in cytoplasm. (A) BrdU-DCX. One DCX-immunoreactive granule cell indicates BrdU uptake. (B) c-Fos-NeuN. c-Fos immunoreactivity was enhanced in many branched brown neurons. (C) Prox-1-CALB. The single- and double-labeled cells can be clearly distinguished. Scale bar: 25 μ m. Abbreviations: BrdU, 5-bromo-2'-deoxyuridine; CALB, calbindin; c-Fos, FBj murine osteosarcoma cellular oncogene; DAB, diaminobenzidine; DCX, doublecortin; NeuN, neuronal nuclei; Prox-1, Prospero Homeobox 1; SSGI, sulfide-silver-gold intensification.

scanning confocal microscopy greatly eliminated this background fluorescence.

Immunohistochemistry for Neuronal Antigens Before AChE Enzyme Histochemistry

If immunohistochemistry followed by the SSGI method was performed before enzyme histochemistry, the cholinceptive neuronal elements vanished. In our pilot experiments, the SSGI method was easily adapted as a post-DAB treatment to AChE histochemistry for the first labeling, but the SSGI with even the mildest power was found to result in unduly intense staining, which means that AChE staining alone usually produced satisfactorily intense labels with high signal-to-noise ratio. The subsequent SSGI was found to thicken the cellular processes artificially and to increase the background slightly, enhancing the signal-to-noise ratio further. Therefore, we sought other colocalization possibilities.

Four sequential versions of immunohistochemistry and AChE enzyme histochemistry were probed to learn about the optimal approach for double staining (see Table 2). The immunohistochemistry for ChAT without SSGI did not interfere with the activity of the AChE (versions 1 and 2). Using hydrogen peroxide treatment was effective to inactivate the HRP, as expected, to prevent further development during the subsequent AChE staining. In version 1, a profuse network of cholinergic fibers appeared in brown, and the perikaryonal sites of the de novo synthesis of AChE after DFP pretreatment of the animals (see AChE pharmacohistochemistry²⁵) were labeled with the distinctive bluish-black DAB-Ni precipitate formed by the catalytic end product (i.e., Hatchett's brown) of the enzyme (Fig. 3A). On the contrary, in version 2, the fine cholinergic fibers, which were originally bluish black, underwent an unintended color conversion during AChE histochemistry and tended to be brown, similar to the cholinceptive neuronal elements, whereas the intense immunoreactive neurons preserved their dark color (not shown). Due to the loss of reliable color contrast between the two kinds of neuronal elements, version 2 is not recommended for use.

Immunohistochemistry for Neuronal Antigens After AChE Enzyme Histochemistry

In our preliminary stainings, the high enzymatic activity of AChE was well preserved in our preparations. Even the mildest version of the subsequent SSGI produced too intense dark signal. Accordingly, alternative post-DAB treatments were tried to replace the SSGI technique, which, simultaneously, were expected to

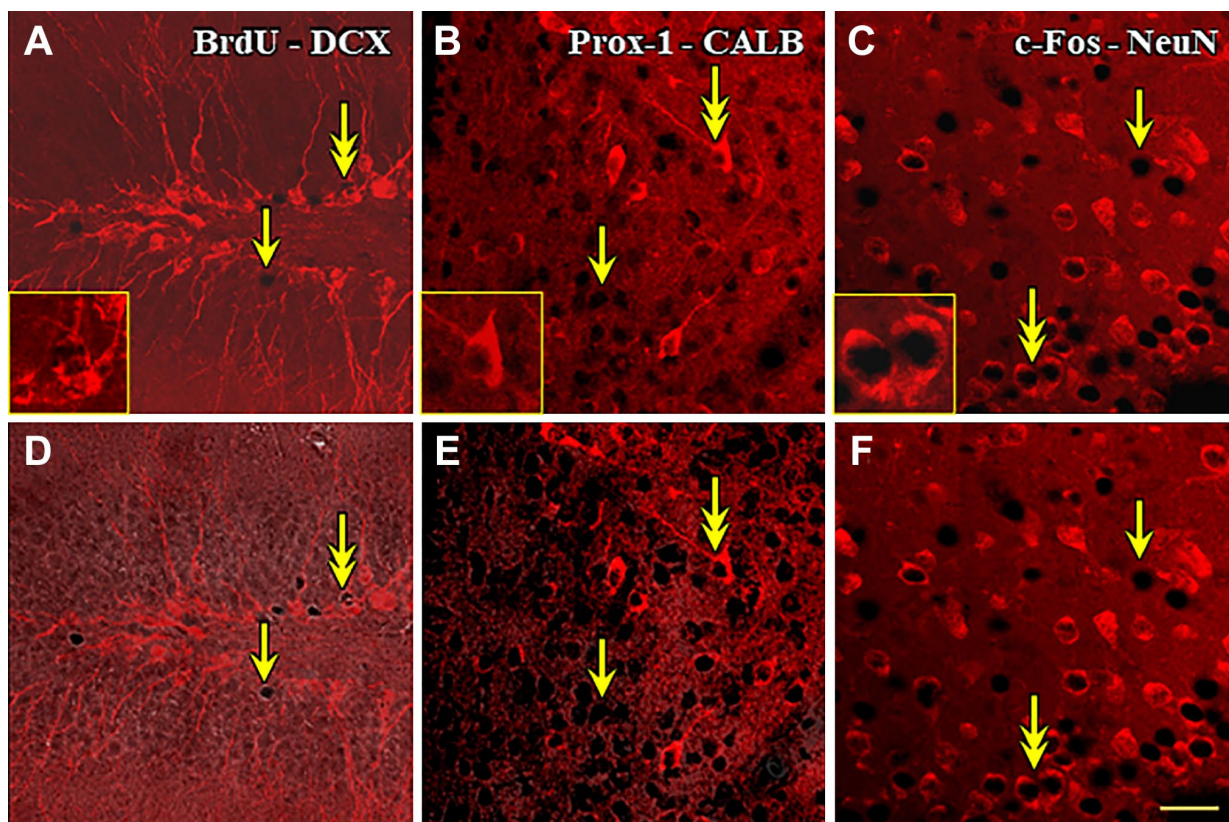


Figure 2. Double immunohistochemical staining for combined bright-field/fluorescence microscopy. The nuclear antigens (BrdU, Prox-1, and c-Fos) were immunostained and enhanced with SSGI similarly as shown in Fig. 1. Thereafter, the perikaryonal markers (DCX, CALB, and NeuN) were visualized with red Cy3-tagged antibodies. Single-head arrows point to immunoreactive nuclei within unlabeled perikarya, whereas the arrows with double heads mark the double-immunoreactive neurons. The same viewfields (A–D, B–E, and C–F) were captured from the sections in two different microscopic modes: laser-scanned confocal (A–C) and combined confocal and phase-contrast (D–F). The marked double-stained cells are magnified in the insets of A to C. Scale bar: 25 μ m. Abbreviations: BrdU, 5-bromo-2'-deoxyuridine; CALB, calbindin; c-Fos, FBJ murine osteosarcoma cellular oncogene; DAB, diaminobenzidine; DCX, doublecortin; NeuN, neuronal nuclei; Prox-1, Prospero Homeobox 1; SSGI, sulfide-silver-gold intensification.

permit the use of bluish-black color of DAB-Ni in the subsequent immunohistochemistry for possibly less antigen density.

In version 3, AChE-positive fibers formed dense network among the CALB-immunoreactive neurons in the brain (Fig. 3B). First, AChE staining was applied, resulting in a bluish-black DAB-Ni polymer, which was then converted to a brown nickel sulfide-containing water-insoluble matrix. This compound precipitate was ultimately covered with extremely stable elementary gold. After the gold toning, the immunoreactive neurons labeled with the bluish-black DAB-Ni polymer can be easily distinguished from the first staining.

In version 4, the perikarya showing synthesis of AChE after DFP pretreatment and the cholinergic

axons were simultaneously visualized in the same section (Fig. 3C). Profound enzymatic reaction was detected in many neuronal cell bodies by deposition of the unintensified brown DAB polymer.

AChE histochemistry was terminated with EDTA treatment, because without this treatment, the color of the perikarya would have undergone a brown-to-black conversion during the visualization of the second neurochemical marker, thereby losing the unambiguous distinction between the sites of the two markers. The application of the EDTA treatment on the sections after the development dissolved the Hatcher's brown, which would otherwise catalyze the DAB polymerization during the immunohistochemical procedure, ended with the bluish-black DAB-Ni precipitation.

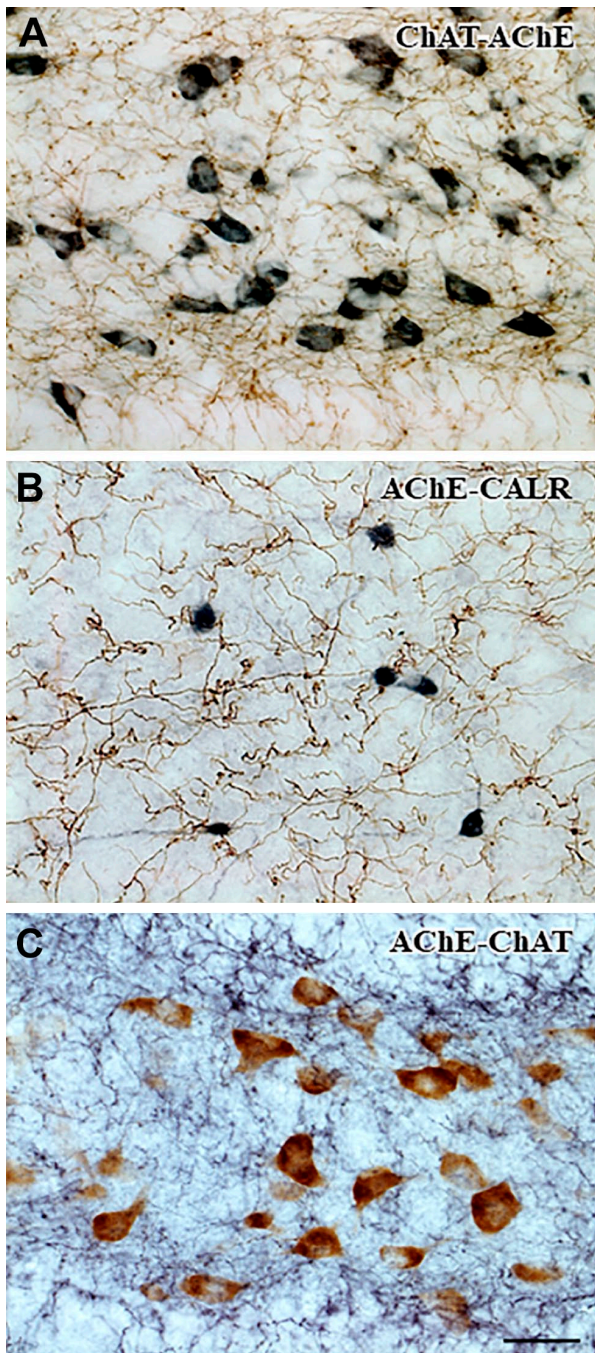


Figure 3. Double labeling with AChE enzyme histochemistry and immunohistochemistry for neuronal markers. (A) ChAT-AChE. Cholinergic fibers were visualized with the brown DAB polymer. The residual HRP activity was quenched with hydrogen peroxide treatment before the subsequent AChE enzyme histochemistry. The perikaryonal sites of the de novo AChE synthesis of the esterase inhibitor-treated animals were labeled with the bluish-black DAB-Ni polymer. Note that no perikaryonal ChAT immunoreactivity is shown. (B) AChE-CALR. AChE-positive

(continued)

Figure 3. (continued) fibers were revealed with fine brown gold deposits over the DAB polymer. Quenching of the reaction end product of AChE ($\text{Cu}_2[\text{Fe}(\text{CN})_6]$) possessing HRP-like catalytic activity was not necessary before the subsequent immunohistochemistry. CALR-immunoreactive cell bodies were developed with the bluish-black Ni-enhanced DAB polymer. (C) AChE-ChAT. The de novo AChE synthesis in the DFP-pretreated animals were labeled with the brown DAB polymer in the neuronal somata. Before immunohistochemistry, the AChE enzyme histochemistry was finalized with EDTA treatment to avoid over-staining and color conversion by the removal of $\text{Cu}_2[\text{Fe}(\text{CN})_6]$. The intense network of ChAT-immunoreactive fibers was labeled with the bluish-black DAB-Ni polymer. Scale bar: 25 μm . Abbreviations: AChE, acetylcholinesterase; CALR, calretinin; ChAT, choline acetyltransferase; DAB, diaminobenzidine; DFP, diisopropyl fluorophosphate; HRP, horseradish peroxidase.

Discussion

Interference in Colocalization Experiments

The elementary steps of the SSGI method⁷ were revised and applied in our present colocalization study. The steps were probed either in different cell populations or in different cell compartments of the identical cell. The common efforts in these consecutive double labeling methods were to terminate the detection of the first marker to prevent any possible interaction with the detection of the second one and to obtain distinct colors representing the sites of the two markers.

Several factors may cause interference between both the consecutive and simultaneous approaches of double labeling techniques. Certainly, the most severe problem is the relative shortage of the available host species of the primary antibodies. On one hand, the researchers' choice is usually restricted to monoclonal mouse/rat and rabbit or polyclonal rabbit/guinea pig and goat/sheep primary antibodies. On the other hand, antibodies to two different antigens are often produced from the same animal species, which hinders the double labeling experiments. Therefore, we tried to elaborate different ways that may allow consecutive labeling of two histological items in the same section.

Double Immunostaining for Light Microscopy

Considering the above-mentioned points, not only double but also multiple labeling approaches can be performed, provided that relatively high cellular concentrations of the markers are present, and the sequence of the detections of the antigens with distinct reaction end product without interference between the immunoreagents and developing conditions is elaborated.

Although three enzymes (HRP, alkaline phosphatase, galactosidase) were found to be potentially suitable for deposition of water-insoluble reaction end products compatible with immunohistochemical procedures,²⁶ HRP seems to be the prevailing enzyme out of them due to its various applicable substrates in different colors. Obviously, DAB is the most widely used chromogen because of the forming polymer's versatile properties: the chelating capacity of some metal ions (e.g., Co, Ni) converting the original brown to blackish hues; osmophilic feature; high insolubility not only in water but also in organic solvents, yielding precise localization of the immunoreactions; and catalytic power of reduction of silver ions in the post-DAB methods also changing the original color.

Using HRP-based double labeling techniques, apparently different approaches are recommended depending on whether the primary antibodies were obtained from the non-cross-reacting animal species (e.g., mouse and goat), allowing simultaneous incubation in the first or even in the second steps of the protocol, or not (e.g., both antibodies are those of mouse). In the first case, the most critical factor is the sequence of DAB polymers in different colors. Using the same immunoperoxidase-based labeling, it was recommended that in the two DAB developments the former one should result in the more intense color including Ni ions, whereas the latter one generates the paler brown DAB.⁴

If the primary antibodies are derived from phylogenetically close species, or even if they belong to the same IgG subclass, the incubations in these immunoreagents must be sequential. To avoid cross-reaction(s) of the detection systems, apparently the researcher may choose between two alternative ways.

The tissue-bound immunoreagents after the first development may be removed by a heat treatment before the subsequent application of the second cycle of antibodies. Elution strategies were elaborated, such as microwave heating or boiling between the two cycles of the visualizations. Microwave treatment was recommended between the applications of the primary antibodies from the same species for colocalizing two different antigens in the same cell of paraffin-embedded tissue.²⁷ Others used traditional boiling for a few minutes to prevent cross-reactions.²⁸ However, these heat-mediated elution techniques were found to be incompatible with the Ni-enhanced DAB polymer by losing its basis color.²⁹ Thus, the first reaction end product was always the DAB polymer, which was followed by elution and subsequent detection of the second antigen by the DAB-Ni polymer. It is worth noting that this approach allows the use of the more intense DAB-Ni polymer as a second signal,

which may be an advantage when the second antigen is experienced to be less detectable, in contrast to the other methods that apply the DAB-Ni polymer as the first signal.

Alternatively, the tissue-bound immunoreagents after the first development may be shielded by atomic silver or gold^{6,7} before the subsequent cycle of antibodies and development. One of the special benefits of this latter way is its compatibility with ultrastructural investigations.^{30,31} This approach also features the optional intensification of the first signal and thereby a higher contrast between the two signals, which can be well utilized when the first signal is in low intensity (e.g., c-Fos in weakly responsive cells). The very intense staining may be also beneficial in statistical analysis of the immunopositive nuclear antigens (BrdU, Prox-1, or c-Fos) in large areas.

Combined Peroxidase and Fluorescence Immunostaining

Immunofluorescence was combined with immunoperoxidase technique to detect two cytoplasmic antigens inside the same cell with primary antibodies produced in the same animal.³² These techniques were tried in both orders. It was found that if the immunofluorescence was used first, it was almost totally quenched by the immunoperoxidase. Thus, the sections after the DAB development required a harsh elution method before the successful application of the fluorescently labeled antibodies, because the intensity of the fluorescence was inversely proportional to that of the peroxidase reaction.

However, in the case of separate intracellular compartments, two antigens were successfully detected using primary antibodies raised in different animal species by means of combining immunoperoxidase and fluorescence techniques without reported cross-talk.³³

Because double immunoperoxidase stainings can be carried out reliably using antibodies from the same species if the first staining is finalized by silver intensification of the DAB polymer,⁶ it prompted us to probe whether immunofluorescence can be also effective on the same specimen after SSGI. The small black immunopositive nuclei were easily spotted at low magnification under transmitted bright-field microscopy, which then could be further investigated for colocalization with another antigen under incident fluorescence microscopy.

DAB was reported to induce nonspecific fluorescence in myelinated nerve fibers and in some perikarya in formaldehyde-fixed nervous tissue³⁴; this background issue can be solved by the appropriate settings of the microscope.

Immunohistochemistry for Neuronal Antigens Before AChE Enzyme Histochemistry

It was reported that the AChE-positive structures were less satisfactorily stained if the immunohistochemistry was performed before enzyme histochemistry.³⁵ However, we did not experience any hindrance in AChE histochemistry, when it was combined with immunohistochemistry, as described here. The SSGI method following the immunoperoxidase was found to be incompatible with the subsequent Tago et al.'s¹⁶ AChE technique using also DAB development: Some component (probably silver ion) of the SSGI technique abolishes the AChE detectability by its enzyme histochemistry. Therefore, another post-DAB treatment had to be found for colocalization of AChE and other neuronal markers.

The HRP of the immunohistochemistry was quenched with conventional hydrogen peroxide treatment. It is worth mentioning that a modified version of the Karnovsky method using cobalt instead of copper ions was elaborated for a simple double staining protocol to receive a reaction end product, which lacks the HRP-like activity.³⁵ However, the cationic exchange appears to result in less precise resolution than the Tago's method.

The approach for the ChAT (revealed by paler DAB polymer) and AChE (revealed by the more intense DAB-Ni polymer) double staining in version 1 was found to be successful, but it did not seem to be optimal, as the less intense DAB polymer would suffice for Tago's AChE histochemistry, including double catalytic reactions first by the enzyme itself and subsequently by Hatchett's brown. However, in version 2, when the polymers were swapped, the clear color distinction was greatly reduced; thus, version 2 was disapproved.

Immunohistochemistry for Neuronal Antigens After AChE Enzyme Histochemistry

Because the widely used Tago's AChE staining marks the distribution of this enzyme reproducibly and intensely, the full power of the SSGI was not necessary to mask the enzyme-containing profile. Our primary purpose of avoiding interactions between the enzyme histochemistry and immunoperoxidase technique was thought to be achieved by means of two different approaches. In one of the protocols, the reaction end product of AChE was shielded by gold plating (see version 3 in section "Combination of Immunohistochemistry and AChE Enzyme Histochemistry"), which was never found to be overstained by the subsequent DAB treatment, but allowed its clear distinction from the DAB-Ni polymer obtained in the second immunoperoxidase step. In the other protocol (version 4), masking of the DAB polymer

was not performed; instead, Hatchett's brown with HRP-like activity was removed by the chelating EDTA to abolish overstaining of the DAB polymer. In both version 3 and version 4, the more intense DAB-Ni polymer was used as a second signal, which can be advantageous if the immunohistochemical process would yield weaker signal.

In our experiences, the thinner fibers require the more intense staining, over the bigger perikarya. Accordingly, we recommend the following ranking of versions 1, 3, and 4 for the fibers or lower density antigens in general: version 3: DAB + Ni + gold > version 4: DAB + Ni > version 1: DAB.

To conclude, double immunoperoxidase protocols require careful tailoring, because of the potential cross-talks between the two systems, mainly due to the cross-reacting antibodies and the color shift of DAB polymer at the second development. In this study, a revised SSGI as a post-DAB treatment after the first development is recommended for parallel detection of nuclear and perikaryonal antigens to resolve these problems. This technique ensures clear distinction between the sites of the two antigens with high signal-to-noise ratio. Our post-DAB method is shown to be compatible with immunofluorescence.

The versatility of our post-DAB reagents is demonstrated by their combinations with AChE histochemistry with the omission of silver intensification. We compared four versions of combinations of immunohistochemistry and AChE enzyme histochemistry, and we recommend two easy methods for double staining of an antigen and AChE. Our sulfide-gold treatment can be possibly adapted for immunohistochemical purposes as well, if one of the target antigens surpasses the other.

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Competing Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author Contributions

ED, IT, EB, and TS designed the study; IT, GS, EP, EB, and TS performed the experiments; IT, GS, and ED wrote the article. All authors approved the final version of the manuscript.

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