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TECHNICAL NOTE

# Visualization of Axonally Transported Horseradish Peroxidase Using Enhanced Immunocytochemical Detection: A Direct Comparison with the Tetramethylbenzidine Method

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**SUMMARY** Visualization of the neuronal tract tracer horseradish peroxidase (HRP) is commonly achieved through the histochemical detection of its enzymatic activity using 3,3′,5,5′-tetramethylbenzidine (TMB) as a chromogen. However, the TMB product is unstable and is incompatible with tissue processing methods that render the enzyme inactive, or when a combination of HRP tract tracing with neuronal phenotype identification is required. In this study we evaluated the applicability of the immunocytochemical detection method for horseradish peroxidase (HRP) visualization using an enhanced detection method based on the Elite ABC peroxidase amplification protocol. The results provide evidence for the immunocytochemical visualization of both anterograde and transganglionic HRP transport in the rat spinal cord. This immunocytochemical method not only showed similar sensitivity to the TMB protocol in detecting HRP-labeled motor neuron perikarya but provided enhanced resolution in the identification of individual neuronal fibers compared to the TMB method. Immunodetection of the HRP tracer also allowed its co-localization with specific neuronal markers using double immunofluorescence techniques. These results offer the first demonstration that sensitive identification of axonally transported HRP can be achieved by immunocytochemistry and provides further support for its use in HRP tract tracing studies. **(J Histochem Cytochem 47:265–272, 1999)**

**KEY WORDS**
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horseradish peroxidase
tetramethylbenzidine
Elite ABC
immunocytochemistry
double labeling
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The enzyme horseradish peroxidase (HRP), either unmodified or lectin-conjugated, is widely used as a reliable and sensitive marker for tracing neural pathways in the nervous system (Waar et al. 1981; van der Want et al. 1997). Axonal transport of HRP occurs both in anterograde and retrograde directions and follows uptake of HRP into cells either via passive endocytosis if free or via active receptor-mediated uptake if conjugated with molecules such as wheat germ agglutinin (WGA) and bacterial toxin fragments (subunit B of cholera toxin, CTB; Wan et al. 1982). Visualization of transported HRP is routinely achieved by histochemical analysis, which localizes the enzyme by its mediated polymerization of certain chromogens that form colored precipitates in HRP-containing cells (van der Want et al. 1997). Among the HRP chromogens, 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 3,3′,5,5′-tetramethylbenzidine (TMB) are commonly used for HRP histochemistry. Several studies have reported that the more convenient DAB method, either alone or intensified with metal salts, lacks the sensitivity of the TMB protocol (Mesulam 1978; Morrell et al. 1981; van der Want et al. 1997). Consequently, the latter protocol is considered the method of choice for detection of axonally transported HRP. However, the TMB method has several limitations, which make the development of improved visualization techniques for axonally transported HRP desirable (Waar et al. 1981). The TMB reaction product is very unstable in ethanol and at neutral pH. Dehydration and counterstaining therefore cause rapid loss of staining. In addition, protocols that inactivate the enzymatic activity

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of the tracer (i.e., paraffin embedding) preclude its visualization by direct histochemistry. Furthermore, co-localization of the HRP tracer with additional antigens by combined immunocytochemistry is complicated by the instability of the TMB product and by the high fluorescent background observed in glutaraldehyde-fixed tissue, a fixative critical for TMB histochemical localization.

Immunocytochemical localization of HRP was recognized early as an alternative method for HRP visualization, particularly useful in cases in which the enzymatic activity of HRP was lost during tissue processing (Vacca et al. 1975). However, the improved sensitivity of this method over the histochemical DAB detection protocol was questioned because of lack of evidence for orthograde enzyme transport (Waar et al. 1981). Since the report by Vacca and colleagues, immunocytochemical methods have evolved significantly, and visualization methods of improved sensitivity have been developed (Hsu et al. 1981; Myers 1988; Ellis and Halliday 1992; Grumbach and Veh 1995). Because the immunocytochemical detection of HRP overcomes the problems associated with TMB detection, it may represent a more appealing procedure for the identification of axonally transported HRP if the sensitivity of this protocol could be enhanced to similar levels to that achieved by the TMB method.

In this study we evaluated the use of an enhanced amplification method for immunocytochemical detection of HRP based on the Elite avidin–biotin–peroxidase complex (Elite ABC; Vector Laboratories, Burlingame, CA). The results indicate that visualization of both anterograde- and transganglionic-transported HRP with the Elite ABC immunocytochemical detection protocol provides similar sensitivity and enhanced resolution compared to the TMB method. Furthermore, exclusion of glutaraldehyde in the fixative solution allowed the use of double immunofluorescence in the detection of tracer–HRP and specific cell phenotype antigens.

## Materials and Methods

### Animal Surgery

Experiments were performed on adult (250–350 g) female Sprague–Dawley rats (Harlan Sprague Dawley; Harlan, TX). The animals were anesthetized with an IP injection of a ketamine (67 mg/kg)/xylazine (6.7 mg/kg) solution before the application of the HRP tracers for both transganglionic and retrograde tract tracing mapping, as described below. After completion of the injections, the muscle layers were sutured, clips were applied to the skin incision, and prophylactic antibiotic treatment was applied topically to the wound. Animals were observed continually after the surgery, and normal body temperature was supported until recovery from anesthesia. The animals were maintained under conditions of controlled light and temperature, and food and water were available ad libitum. Institutional Animal Care and Research Advisory Committee regulations were observed for surgical and care procedures.

### Transganglionic Tract Tracing

Five animals were used for transganglionic HRP labeling. The right sciatic nerve was surgically exposed at the midthigh level in these animals and a strip of parafilm was inserted temporarily under the nerve to prevent leakage of the tracer into the surrounding tissue. Using a Nanoject injector (Drummond Scientific; Broomall, PA.)-driven glass micropipette, 4–7 μl of an HRP mixture (20% HRP Type IV; Sigma Chemical, St Louis, MO/5% WGA–HRP; Sigma Chemical/0.2% B-choleratoxin–HRP; List Biological Labs, Campbell, CA/5% dimethylsulfoxide) was directly injected into the nerve 0.5 cm distal from its emergence from the great sciatic notch. The micropipette was left in place for 5 min after injection to allow diffusion of the tracer before closing the wound. B-choleratoxin HRP was omitted from the HRP mixture in two animals to allow the specific visualization of unmyelinated C-fibers in the lamina II of the dorsal horn of the spinal cord (LaMotte et al. 1991).

### Anterograde Tract Tracing

Hemilaminectomies were performed in three separate animals at the T13–L2 vertebral segments to expose the L4–L5 dorsal roots. The isolated roots were placed on a parafilm strip and transected by crushing the roots twice for 10 sec each, using 0.5-mm jewelers' forceps, taking care not to disrupt the perineurium. The peripheral end of the root was ligated with 6-0 suture to facilitate the preferential diffusion of the tracer towards the spinal cord. Using a Nanoinjector-driven micropipette, 4 μl of the HRP mixture was delivered over a 2-min period directly into the lesioned area of each dorsal root. The micropipette was left in place for 2 min after injection to allow tracer diffusion.

### Histochemistry and Immunocytochemistry

At the end of the treatment period (i.e., 24 hr for anterograde labeling and 48 hr for transganglionic tract tracing), the animals were anesthetized and perfused transcardially with either 1% paraformaldehyde/1.5% glutaraldehyde or 4% paraformaldehyde in buffered saline. The lumbar spinal cord was then removed and postfixed for at least 24 hr at 4C. Coronal tissue sections were obtained either at 10 μm using a cryostat (Hacker-Bright Instrument; Huntingdon, UK) or 50 μm using a Vibratome (Lancer; Technical Products International, St Louis, MO). The tissue sections were divided into five alternate sets at 250-μm and 50-μm intervals for microtome and cryostat sections, respectively, and were either processed immediately or stored in cryoprotectant solution (Watson et al. 1986) at −20C until processed.

### The TMB Method

Visualization of HRP was performed according to the protocol described by Mesulam (1978). Briefly, the tissue was incubated in a 0.005% TMB/0.05% sodium nitroferricyanide/

0.2 M acetate buffer (pH 3.3) solution for 20 min at 4C before addition of the hydrogen peroxidase (0.03% final concentration) and was then incubated for 20 min. This method was optimized according to Mesulam and colleagues (1980) to achieve the maximal sensitivity compatible with minimal size of nonspecific TMB crystal deposition. A commercially available TMB liquid substrate system containing the chromogen, buffer, and hydrogen peroxidase (Sigma) was also tested, but it rendered less satisfactory results compared to the described protocol. The sections were mounted on gelatinized slides, air-dried for 4 hr, and dehydrated through serial ethanol dilutions (10 sec at each dilution) before being coverslipped with Permount for light microscopic examination.

### Anti-HRP Immunocytochemistry

After removal of residual fixative from the tissue sections by extensive PBS, pH 7.5, rinses, the sections were incubated in 3% hydrogen peroxidase to quench endogenous peroxidase activity. After further rinsing, the tissue was then incubated with 5% normal goat serum (NGS) to reduce nonspecific staining and subsequently incubated in a rabbit polyclonal HRP antiserum (1:20,000; Sigma) solution for 24 hr at RT with continuous agitation. Visualization was achieved by tissue incubation in biotinylated goat anti-rabbit IgG secondary antibodies (1:600). Biotin-labeled tissue was further processed using the Vectastain Elite ABC reagents and was developed with a solution of hydrogen peroxide (0.003%) and diaminobenzidine (0.02%). The specificity of the HRP antiserum was corroborated by the lack of staining in tissue in which the primary antibody was excluded from the staining protocol. In addition, the localization of transported HRP was completely abolished when the HRP antiserum was preabsorbed with HRP ($1 \times 10^{-6}$ M, Type IV; Sigma) 24 hr before its use for immunocytochemistry. Tissue sections of both control and experimental groups were simultaneously developed in identical incubation solutions. Sections were mounted on gelatinized slides and coverslipped for microscopic evaluation.

### Double Immunofluorescence

After incubation of the tissue with 5% NGS, the sections were incubated simultaneously with the rabbit anti-HRP (1:4000) and monoclonal antiserum for the nonphosphorylated form of neurofilament H (MSI-32; 1:500; Sternberger Monoclonals, Lutherville, MA). Visualization of the primary antibodies was achieved by simultaneous incubation in Texas Red-labeled goat anti-rabbit and fluorescein-labeled goat anti-mouse (1:250 for both antibodies; Jackson ImmunoResearch Laboratories, West Grove, PA). The sections were mounted on slides and coverslipped with 5% *N*-propyl-gallate in glycerol and were visualized by epi-illumination.

## Results

### Transganglionic Tract Tracing

After injection of an HRP/WGA–HRP solution to the sciatic nerve, axonally transported HRP was localized preferentially in lamina II of the dorsal horn (Figures 1A and 1B) and in the motor neurons of the ventral horn (Figures 1C–1F). Identification of HRP-containing fibers and cell bodies in these areas appeared qualitatively similar with either TMB (Figures 1A, 1C, and 1E) or immunocytochemical (Figures 1B, 1D, and 1F) visualization. Visualization of the HRP by the TMB method, however, also resulted in moderately high background, characterized by nonspecific deposition of TMB crystals over the tissue (Figures 1A and 1C). Nonspecific staining was also observed in blood vessels, indicating the presence of residual erythrocytes. In contrast, immunodetection with the enzyme resulted in very specific staining of the labeled neuronal fibers and cell bodies, with minimal background and no detection of immunoreactivity in other types of cells (Figures 1B, 1D, and 1F). Identification of labeled ventral motor neurons was similarly achieved by the two HRP detection methods, as indicated by the comparable number of stained perikarya (Figures 1C and 1D). Conversely, visualization of individual motor neuron processes, such as axons or dendrites, appeared to differ between the two visualization methods. The granulated nature of the TMB staining yielded discontinuous labeling of axons and dendrites which, along with the highly granular background, made the identification of individually labeled processes a challenging task. This was particularly evident when visualization of individual labeled fibers distal from the neuron perikarya was attempted (Figure 1E). In contrast, the use of anti-HRP antibodies for HRP detection permitted the identification not only of the labeled cell bodies but also of the HRP-containing neuronal processes (Figure 1F), suggesting that the resolution achieved by the immunocytochemical method is greater than that of the TMB protocol.

### Anterograde Tract Tracing

Application of the lectin-conjugated HRP mixture to the dorsal roots resulted in the labeling of sensory axons in the spinal cord as detected by both the TMB (Figures 2A and 2C) and the anti-HRP (Figures 2B and 2D) method. However, the two protocols differed in the qualitative signal-to-noise ratio and consequent resolution in HRP detection. The nonspecific background was higher in the TMB-processed tissue, in which TMB crystals of moderate to large size were found throughout the tissue. Evaluation of the traced axons at higher magnifications further emphasized the difference in signal-to-noise ratio yielded by the TMB and the anti-HRP protocol (Figures 2B and 2D). Whereas the TMB visualization of HRP-labeled axons was granulated, discontinuous, and obscured by nonspecific deposits of TMB crystals (Figure 2B), the immunocytochemically stained fibers were clearly and more specifically defined (Figure 2D). In addition, the num-

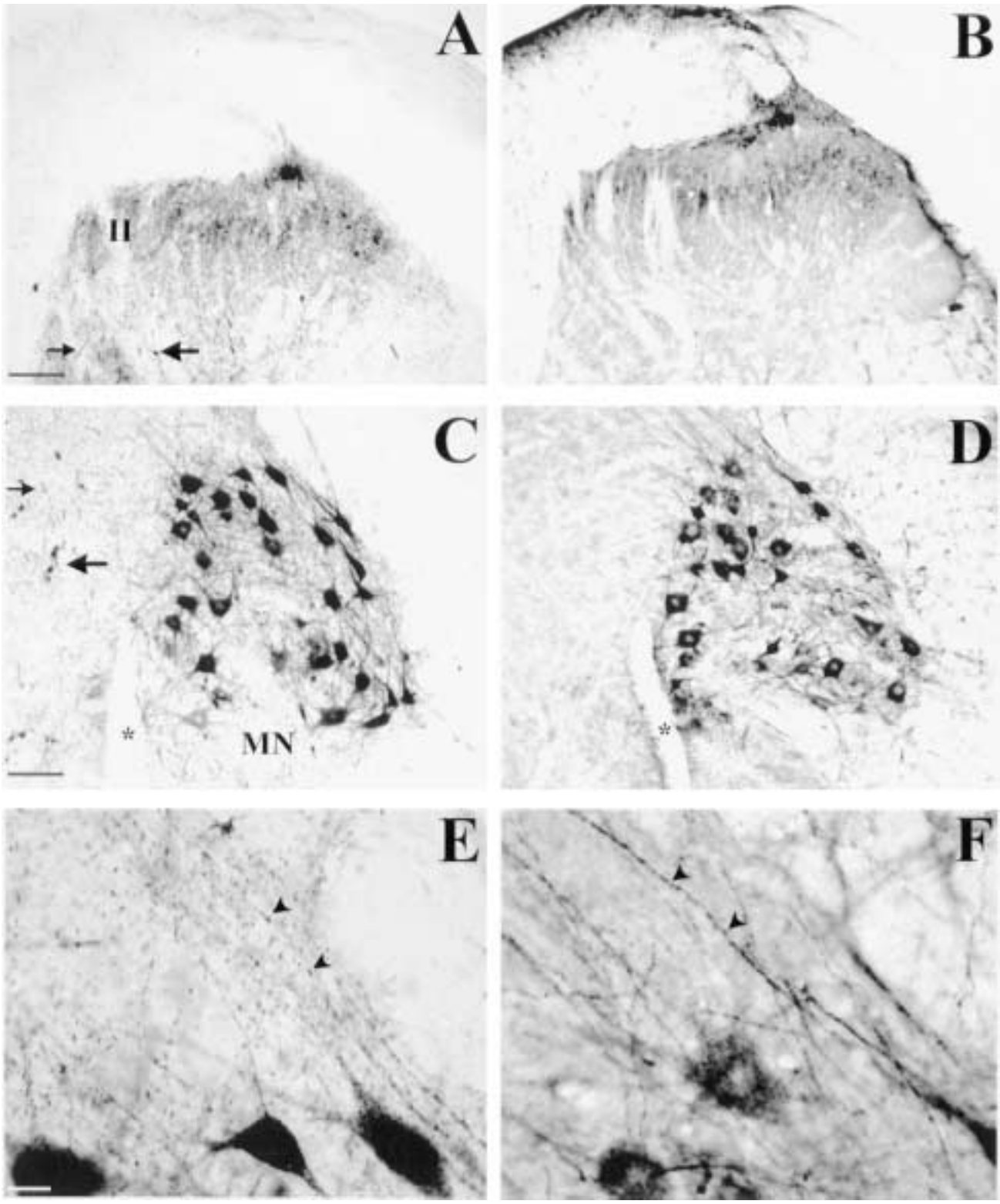


**Figure 1** Visualization of transganglionically transported HRP in the rat spinal cord. Photomicrographs are of immediately adjacent coronal sections from the same animal (asterisks denote knife mark in the ventral horn) processed for HRP detection using either TMB histochemistry (**A,C,E**) or ABC Elite immunocytochemistry (**B,D,F**). The two methods rendered qualitatively similar colorimetric localization of the tracer both in sensory afferents to lamina II (II) of the dorsal horn (**A,B**) and in the perikarya of ventral motor neurons (**C,D**). Bars = 100 μm. In contrast, the identification of motor neuron fibers (arrowheads) by TMB was complicated by its granular and discontinuous ap-

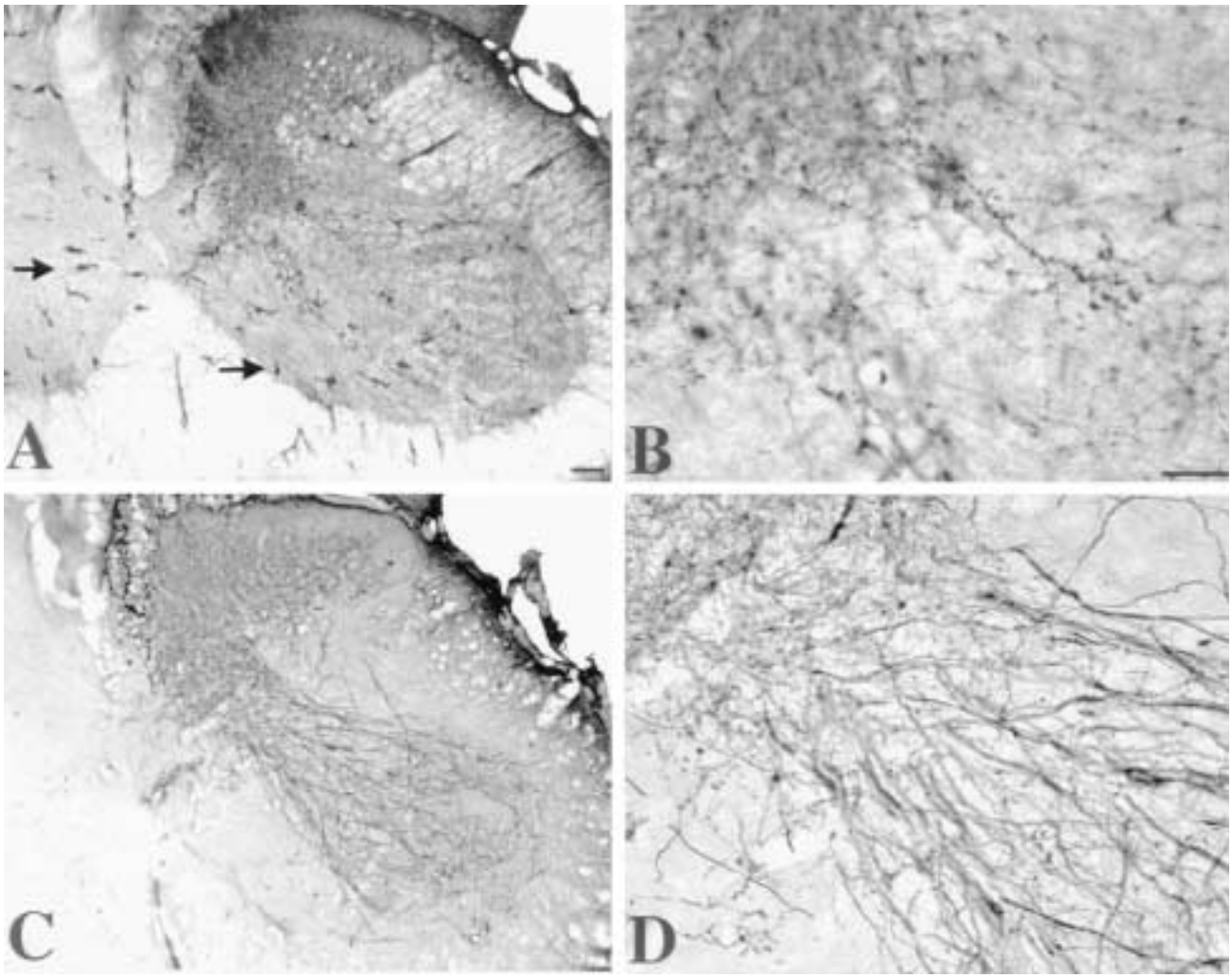


**Figure 2** Anterograde HRP tract tracing. Photomicrographs illustrate the identification of primary sensory afferents in adjacent spinal cord sections by either the TMB (**A,B**) or the immunocytochemical (**C,D**) protocol. Visualization of sensory axons is notably enhanced by the immunocytochemical method, not only in facilitating the tracing of individual fibers but also in the apparent number of detected axons (**D**) compared to that achieved by the TMB method (**B**). Nonspecific TMB crystal depositions (**A**, arrows) are obviated by the immunodetection protocol (**C**). Bars = 100 μm.

bers of labeled fibers appeared to be increased in sections processed immunocytochemically (compare Figures 2B and 2D).

### Simultaneous Visualization of Axonally Transported HRP and Cell-specific Markers

Figure 3 illustrates the simultaneous visualization of axonally transported HRP (Figure 3A) and the neurofilament neuronal cell marker (Figure 3B) in the ventral horn of the spinal cord by double immunofluorescence. Co-localization of the two markers in the perikarya (arrows) and neurites (arrowheads) of ventral motor neurons demonstrates the ability of the immunocytochemical method for the simultaneous identification of the tracer with specific cell phenotype markers in these cell compartments.

pearance (**E**), whereas appreciation and tracing of fine dendrites and axons were facilitated by the immunocytochemical method (**F**). Bar = 20 μm. Nonspecific staining, both as randomly distributed crystals (small arrows) and cells with apparent endogenous peroxidase activity (large arrows) was produced only by the TMB method (**A,C**).

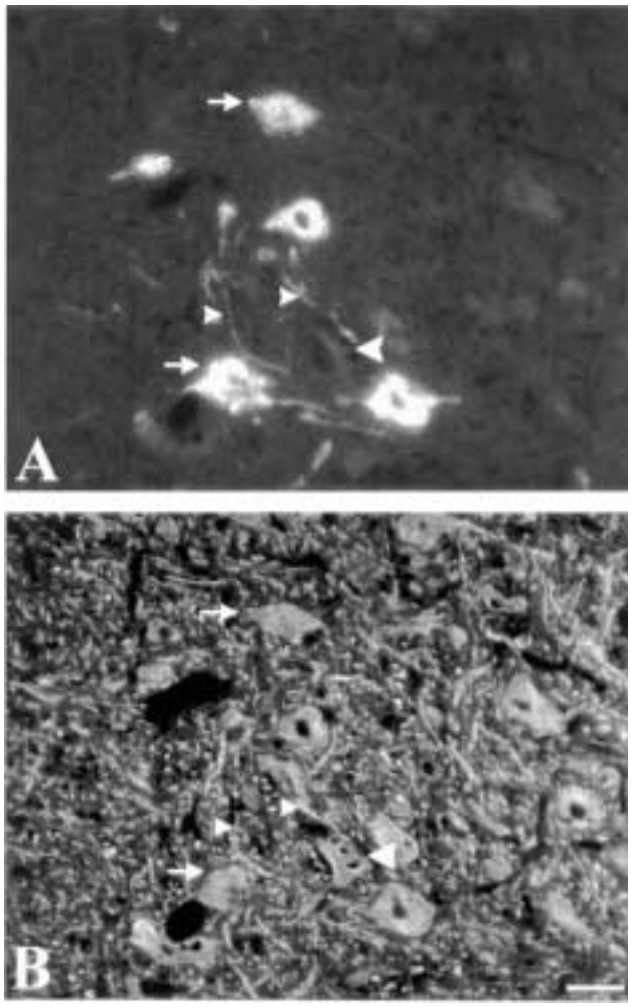


**Figure 3** Simultaneous identification of the HRP tracer and neuronal phenotype markers by double immunofluorescence. Photomicrographs illustrate the co-localization of transganglionically transported HRP (**A**) and neurofilaments (**B**; indicative of neuronal phenotype) in both the perikarya (arrows) and neurites (small arrowheads) of ventral motor neurons in the spinal cord. Large arrowheads indicate a motor neuron not labeled by the HRP tracer. Bar = 20 μm.

## Discussion

Unmodified and lectin-conjugated HRPs are widely used as reliable and sensitive markers for tracing neural pathways (Waar et al. 1981). Detection of the axonally transported HRP is commonly performed with the TMB method because of its reported greater sensitivity over that of other chromogens (Mesulam 1978; Morrell et al. 1981). However, practical application of the TMB protocol is hampered by several unfavorable characteristics, and this protocol is particularly incompatible in circumstances of enzyme inactivation during tissue processing or when the combination of HRP tract tracing with cell phenotype identification is required. Detection of the HRP tracer through the use of specific antibodies circumvents the problems associated with the TMB method and allows HRP visualization under conditions of enzyme inactivation (Vacca et al. 1975; Schmidt and Trojanowski 1985) or in combination with immunocytochemical labeling (Lindh et al. 1989). The immunocytochemical method for axonally transported HRP detection was first proposed more than two decades ago as a sensitive alternative to histochemical methods for HRP visualization (Vacca et al. 1975). However, its utility was undermined by the lack of evidence in that early study for orthograde enzyme transport (Waar et al. 1981). Despite the many advantages of the immunodetection protocol over the TMB method in HRP visualization, the latter method is regarded as the method of choice in localization of axonally transported HRP (Mesulam 1978; Morrell et al. 1981; Schmidt and Trojanowski 1985; Lindh et al. 1989).

In this study we have reevaluated the applicability of the immunocytochemical detection method using a more recent and enhanced detection method based on the Vector Elite ABC peroxidase amplification protocol. This method is based on equimolar ratios of avidin D and biotinylated peroxidase (Grumbach and Veh 1995) and generates a considerably higher signal intensity when compared to conventional ABC protocols (Ellis and Halliday 1992).

### Detection of Axonally Transported HRP

Our results provide evidence for the immunocytochemical visualization of both anterograde and transganglionic HRP transport in the rat spinal cord. Anterograde transport was observed after injection of the HRP tracer into the primary dorsal afferents and subsequent visualization of the enzyme in the dorsal and ventral horns of the spinal cord. Transganglionic HRP transport was verified by visualization of the HRP tracer in the dorsal root ganglia (not shown) and in both primary sensory afferents, as well as in ventral motor neurons, after injection of the tracer into the sciatic nerve. In all cases evaluated, the sensitivity of the immunocytochemical method for visualization of the HRP tracer was at least qualitatively similar to that obtained by the TMB method. This is clearly indicated by the apparently equal number of ventral motor neurons identified by the two methods.

### Enhanced Resolution of HRP Visualization by Immunocytochemistry

Visualization of axonally transported HRP by the TMB method, although sufficient for localization of labeled perikarya, is unsatisfactory for identification and tracing of neuronal fibers. The identification of

neurites by TMB is complicated by the granular and discontinuous nature of the TMB precipitation product, which limits the appreciation and tracing of fine dendrites and axons. The nonspecific deposition of TMB crystals on the surface of tissue sections further obscures the detail of the labeled pathways. Conversely, the immunocytochemical method allows detailed identification of neurites and facilitates the tracing of individual neuronal fibers. This observation most likely reflects the differences between the DAB and the TMB chromogens. In contrast to the granular nature of the TMB precipitate, the DAB product is noncrystalline and therefore diffuses freely through the fibers, allowing more even and continuous labeling. In addition, discrimination of individual labeled fibers was facilitated by the low nonspecific staining achieved by the immunodetection protocol. Suppression of endogenous peroxidase activity and the use of low titers of anti-HRP antibodies (i.e., 1:20,000) reduced the nonspecific staining compared to the TMB method, thereby enhancing the signal-to-noise ratio.

### Co-localization of the HRP Tracer and Cell Phenotype Markers

The incompatibility of the TMB method with immunocytochemical determination of cell phenotype has limited the amount of information obtained from tract tracing studies using this histochemical procedure for HRP visualization. Therefore, the identification of transmitter phenotype of traced or innervated neurons, synaptic contact establishment by the traced cells, and/or neuronal activity, as indicated by functional markers, requires alternative approaches. Such methods either can include additional steps in tissue processing to stabilize the TMB reaction product (Rye et al. 1984) or can use different HRP detection strategies, such as that based on the amplification of biotin sites by the HRP-mediated deposition of biotinylated tyramide (Kressel 1998). However, these techniques are limiting and/or demanding, because many and/or special steps are required for optimal deposition and preservation of the enzymatic product (Mesulam 1978; Mesulam et al. 1980; Morrell et al. 1981; Rye et al. 1984; Adams 1992; van der Want et al. 1997; Kressel 1998).

In this report we have demonstrated that co-localization of the HRP tracer and cell phenotype markers can be achieved by double immunofluorescence. Although the immunofluorescence technique required a higher concentration of the anti-HRP antiserum because of its reduced sensitivity compared to the ABC detection systems, this method allowed the identification of transganglionically labeled motor neurons in the spinal cord with visualization of both perikarya and proximal neurites.

Taken together, these results provide further support for the immunocytochemical detection of HRP tracer as a sensitive alternative for the visualization of axonally transported HRP and the applicability of this method for the simultaneous identification of cell phenotype markers.

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