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SHORT COMMUNICATION

Counterstaining Improves Visualization of the Myenteric Plexus in Immunolabelled Whole-Mount Preparations

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Abstract Immunocytochemistry has emerged as a powerful research tool in neurobiology. One of the widely used methods is an indirect fluorescence technique that uses FITC-conjugated IgG to visualise protein expression within tissues, but a major drawback of this technique is the high background fluorescence due to non-specific antibody binding. Gut innervation is complex and best visualized in three-dimensions in whole mount preparations. We describe a simple and easy to use counterstaining procedure in conjunction with an indirect immunofluorescence technique in gut whole mount preparations that largely eliminates background fluorescence and creates a contrasting background against the bright antigen-antibody complexes. Furthermore, this technique allows the detailed qualitative and quantitative study of myenteric plexuses in whole-mount preparations.

Keywords Immunocytochemistry · Indirect fluorescence · Counterstaining · Myenteric plexuses · Whole mount preparations

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Introduction

The myenteric plexuses (MP) of the mammalian enteric nervous system have a complex arrangement of ganglionated neural plexus that may be difficult to appreciate on thin sections. The whole mount preparation (WMP) is an elegant technique for visualizing these plexuses as it gives a three-dimensional morphological view of the meshwork of fibers [1–3]. Furthermore, the neurons of the MP can be identified by staining for the presence of neurochemicals that include neuropeptides and neurotransmitters [4–7], thus permitting quantitative and qualitative estimates of cells in tissue. One of the more widely used methods for visualization of MP is indirect fluorescence, in which a secondary antibody tagged with a fluorescent marker is used to visualize the antigen-antibody complex [7, 8]. However, this technique often produces non-specific background fluorescence that interferes with clear visualization of antigen-antibody reactions and makes interpretation difficult. Counterstaining is one way to overcome this, as the bright fluorochromes are viewed better in relation to the dark background. Here we describe a simple and easy to use counterstaining technique that can be used in WMP to better visualize MP in conjunction with indirect fluorescence.

Methodology

Tissue preparation

The research project was approved by the local committee of the ethics on animal experiment and the animals were cared for in accordance with institutional guidelines. The fetuses of timed-pregnant Sprague-Dawley rats were collected by cesarean section on gestational day 21, immediately before

term and were killed by decapitation. The anorectum of each fetus was dissected free of surrounding tissues under magnification, opened longitudinally, stretched flat and pinned on a Silgard Petri Dish. The tissues were fixed overnight at 4°C in Zamboni Solution {4% formalin, 0.2% picric acid in 0.1 mol/l phosphate buffer (pH 7.3)} and then stored dry at −80°C.

The fixed specimens were cleared through three changes of Dimethyl Sulfoxide (DMSO) {Sigma-Aldrich, USA} (10 min each), followed by three 10 min washes in phosphate buffered saline (PBS) {10-PBS tablets (Oxoid Australia Pty Ltd) dissolved in 1-L distilled water} before dissection under magnification on a contrasting background. The proximal and distal ends were marked with oblique and transverse cuts respectively and the mucosa was separated in one layer from the muscularis mucosa. The circular muscle fibers were gently peeled off using high power magnification, leaving behind the MP attached to the longitudinal muscle layer.

Immunocytochemistry

Neurochemicals Neuron Specific Enolase (NSE) and Vasoactive Intestinal Peptide (VIP) were detected in the fixed tissues by immunocytochemistry using commercially available primary antibodies. Simultaneous negative controls were generated for each antibody by omitting primary antibodies. All steps were conducted at ambient temperature with incubation carried out in a humid chamber. After blocking non-specific binding sites with 10% normal goat serum in PBS −0.1% Triton X-100 for 1-hour, the tissues were given three 15 min PBS washes with constant rotation before being incubated with a primary antibody, NSE (Chemicon International, CA, USA) diluted 1:100 in 1% normal goat serum or VIP (Accurate Chemical & Scientific Corporation, NY, USA) pre diluted (1:4) for 18–24 h. Following another three 15 min washes with PBS, the tissues were incubated in the dark with goat anti-rabbit fluorescein isothiocynate (FITC) conjugated IgG (Chemicon International, CA, USA), diluted 1:100 in 1% goat serum in PBS for 90 min. To remove unbound antibody, the tissues were given three 20 min washes (with constant rotation) and then mounted on glass slides in Dabco anti-fading mounting media. This was prepared in our laboratory by dissolving 1 g of Dabco (1,4-Diazabicyclo {2.2.2} octane (Sigma-Aldrich, USA) in 36 ml of Glycerol + adding 4 ml of PBS after heating. The mounted tissues were then examined under a fluorescence microscope (Epi-Fluorescence Microscope, Olympus BX-40, Japan) 100 W Burner, 488 nm excitation filter and 520 (± 10) nm bp Barrier filter, and the photographs were taken with an attached digital camera (Olympus Camedia C-40, Japan).

Counterstaining procedure

Methyl Green (E-Merck Darmstadt, CI Nr. 42585, S. Nr. 788) and Eriochrome Black T (Sigma-Aldrich, USA, Cat # E2377) were used to counterstain the WMP. A stock solution of methyl green (0.8% w/v in distilled water) was diluted 1:9 (v/v in PBS) to give a working solution whereas the stock solution of Eriochrome Black-T (1.65% w/v in distilled water) was diluted at a ratio of 1:1 in PBS. The specimens to be counterstained were treated with methyl green for a 3-minute period and then given two 5 min washes in PBS with constant rotation. Each specimen was then dipped into the Eriochrome Black-T for 10–15 s (depending on the thickness of the tissue), followed by three more PBS washes. Each sample was counterstained individually.

Results

A high background of non-specific green fluorescence in the nerve cells and fibers of fixed tissues incubated with antibodies to NSE and VIP was observed in the tissue samples that were not counterstained and which significantly compromised visualization of the myenteric plexuses (Fig. 1A). This background persisted, despite our attempts to optimize the assay by conventional means. After counterstaining with Methyl Green and Eriochrome Black T, the background cells fluoresced red and provided marked contrast between the brilliant green immunoreactive cells and nerve fibers against a reddish-brown background, thus enhancing visualization of myenteric plexuses in WMPs (Fig. 1B). The negative controls for NSE and VIP are devoid of stains (Fig. 1C).

Discussion

One of the problems in immunocytochemistry is non-specific binding of immunoglobulin or other proteins, which makes it difficult to detect specifically stained cells in the context of surrounding cells. Counterstaining in conjunction with immunofluorescence is an effective measure to reduce background staining [9–11]. Various techniques of counterstaining have been described in thin section studies. Nicholas and McComb first reported that counterstaining with Evans blue greatly reduced unwanted fluorescence [12]. Since then, this method has been used extensively in a variety of immunofluorescence techniques [13–15]. Toluidine blue and propidium iodide counterstaining have also been reported to be effective in immunofluorescence studies [16, 17]. In our study, we initially tried to reduce non-specific binding using conventional techniques, including dilution of reagents, absorption of conjugate with tissue powder and titration of antibodies.

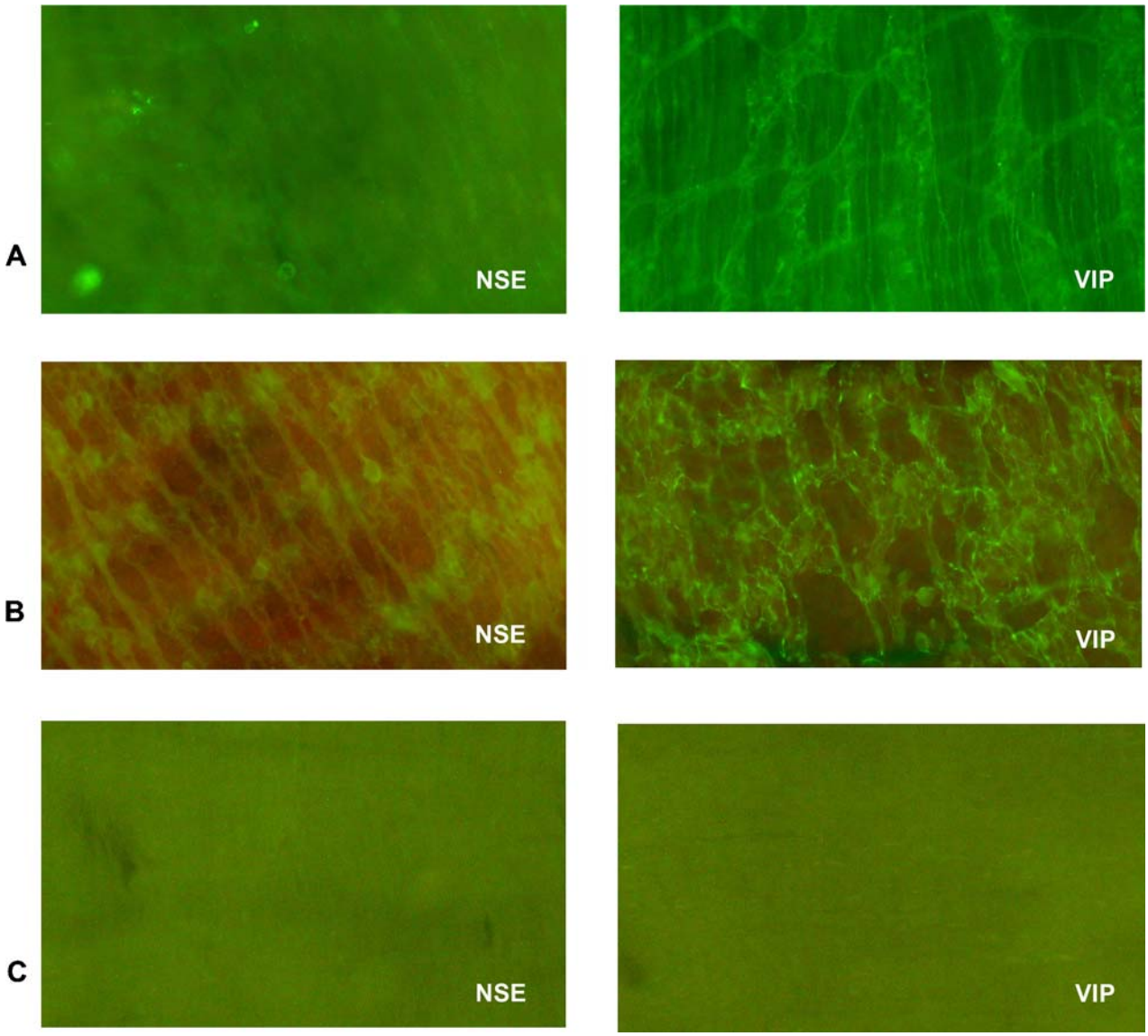

Fig. 1 Immunocytochemical staining of whole mount preparations of the myenteric plexuses of rat anorectum following incubation with rabbit antibodies specific to NSE and VIP. A fluorescent-labeled anti-rabbit IgG antibody was used to detect primary antibody binding. (A) *Before counterstaining* – this method gives high background staining making the interpretation of neurons and nerve fibers difficult (*Magnification = X400*). (B) *After counterstaining* – immunoreactive cells stain brilliant green against a reddish-brown contrasting background (*Magnification = X400*). (C) *Negative Controls* – No staining of neurons and nerve fibers (*Magnification = X400*)

However these methods failed to completely overcome non-specific binding. Likewise, counterstaining WMPs in 0.1% Evan blue (as reported previously [18]), failed to improve specificity of the assay. This motivated us to develop a counterstaining method that would improve sensitivity and specificity when immunofluorescence is used to visualise the myenteric plexus in WMPs.

In developing this method, we took into account the need for a water-soluble counterstain that would be effective at neutral or basic pH and would not mask the green fluorescence of the FITC-conjugated secondary antibody. Methyl green, a specific stain for deoxyribonucleic acid, has been used as a counterstain in fluorescence microscopy since 1967 and results in red fluorescence [19], whereas Eriochrome Black, originally used as an aluminium chelate, produces a uniform reddish brown background with no apparent masking of specific FITC staining [20]. These two dyes, which produce these effects by different mechanisms, offer better color contrast (red fluorescence) against the FITC fluorescence (green). Moreover, the dyes reduce the non-specific staining by competing for the same structures on the tissues that are responsible for the binding of immunoglobulins by their Fc-portion, which is composed only of heavy chains at the carboxyl terminal (the base of the Y) of the Ig molecule.

The neurons of the MP in the WMPs counterstained with the combination of these two dyes can be observed with a clarity that facilitates the qualitative and quantitative analysis of the tissues. The plexuses, neurons and fibers are consistently and uniformly visible in each specimen stained with anti-NSE and anti-VIP antibodies. In some specimens, at sites, a gap between the plexuses was observed; this was not due to the concealment of nerve cells by counterstains, but instead was because of loss of the MPs during microscopic dissection. In general, the high neuronal density in MP can be appreciated clearly with the counterstained specimens, making this procedure valuable in the quantitative assessment of these neurons. This counterstaining method also provides a good three-dimensional panoramic view. The similar analysis of preparations that do not use these counterstains is not possible.

In summary this is a simple and easy to use counterstaining technique that enhances the ability to study nerve fibers and neurons in whole mounts, and facilitates detailed qualitative and quantitative analysis of the MP.

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