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Reciprocal Nerve Staining (RNS) for the concurrent detection of Choline Acetyltransferase and Myelin Basic Protein on paraffin-embedded sections

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Abstract

Background.—Objective of our work was to develop a sequential double nonfluorescent immunostaining method which allows the selective identification of myelinated motor fibers in paraffin-embedded samples of peripheral nerves. Motor recovery after a nerve gap-lesion repaired by artificial nerve-guides (“conduits”) is often less complete and slower than sensory recovery. The mechanism for this is not fully understood.

New method.—Incubation in sheep polyclonal choline acetyltransferase antibody (Abcam 18736) at dilution of 1:150 was followed by incubation in mouse monoclonal anti-myelin basic protein antibody (Abcam 62631) at a dilution of 1:5,000. Counterstaining was performed with hematoxylin QS (Vector Labs H-3404).

Results.—Immunostaining of choline acetyltransferase and myelin basic protein can be combined together and results show a good contrast between the light brown of the choline acetyltransferase reaction product and the green of myelin basic protein reaction product. Cell nuclei are stained blue.

This new protocol retains the advantages of paraffin embedded sections such as (i) having a relatively simple methodology, (ii) years-long storage life, and (iii) easy sharing among laboratories.

Comparison with existing method.—This specific combinatorial protocol has never been used before on paraffin embedded sections. It has been named “reciprocal nerve staining” (RNS).

Conclusions.—Routine combination of choline acetyltransferase and myelin basic protein immunostaining provides a highly specific, highly contrasted paraffin-embedded sections where optical differentiation of myelinated motor fibers is easy and straightforward. This method will

likely simplify and speed-up the routine histological study of nerve regeneration and will contribute a better identification of the nerve motor component.

1. Introduction

The objective of our work was to develop a sequential double nonfluorescent immunostaining method which allows the selective identification of myelinated motor fibers on paraffin-embedded samples of peripheral nerves. Motor fibers in peripheral nerves are cholinergic and myelinated. The immunostaining of choline acetyltransferase (ChAT) is used to detect nerve fibers whose main neurotransmitter is acetylcholine, as found in peripheral nerve motor fibers. The immunostaining of myelin basic protein (MBP) is used to differentiate between myelinated and unmyelinated nerve fibers. Both immunostaining methodologies have been largely employed in association with fluorescence microscopy, where samples are required to be stored at low temperature to prolong the operative life of the fluorescent secondary antibody. These immunostaining techniques have been individually applied to paraffin-embedded samples too (1–8). However, to the best of our knowledge, they have never been combined on paraffin embedded sections and routinely applied in a published research study. We wanted to exploit the advantage of paraffin embedded sections of having a relatively simple methodology, years long storage life and easy sharing among laboratories worldwide. The method that we developed has been named “reciprocal nerve staining” (RNS).

2. Materials and Methods

Sixteen New Zealand White male Rabbits were used for the study of the nerve-guide assisted sciatic nerve regeneration following a complete monolateral gap-injury. Thus, the present report study included experiments with sixteen pairs of samples, each consisting of a set of proximal-distal stumps of a lesioned sciatic nerve and the contralateral intact sciatic nerve. All these pairs were used to develop and test the RNS. The experiments were conducted under the Rutgers University Institutional Animal Care and Use Committee (RU-IACUC) approved protocol 10–005. Consecutive sections of each sample were subjected to four additional staining procedures, applied by using standard protocols: routine hematoxylin/eosin staining; Masson trichrome staining; single ChAT immunostaining; single MBP immunostaining.

In our RNS on-slide protocol, sections were deparaffinized, dehydrated through a graded ethanol series, and subjected to heat-induced epitope retrieval with citrate buffer, pH 6.0 for 20 minutes at 98 degrees Celsius using a pressure cooker. The sections were blocked with 10% normal donkey serum for 30 minutes followed by 48-hour incubation in sheep polyclonal antibody to choline acetyltransferase (Abcam 18736) at a dilution of 1:150. Incubation in the secondary antibody (Ultra Polymer Donkey anti Sheep IgG (H&L) HRP, Immunoreagents inc., lot # M-01–163-P1, no dilution necessary, 30 minutes at room temperature) was followed by reaction for 5 minutes with DAB chromogen substrate (Vector Labs SK-4105). After this first part of the double immunostaining, slides were again subjected to heat-induced epitope retrieval with citrate buffer, pH 6.0 for 20 minutes at 98 degrees Celsius using a pressure cooker. Next, the sections were blocked with 10% normal

horse serum for 30 minutes followed by 1 hour of incubation with mouse monoclonal anti-myelin basic protein antibody (Abcam 62631) at a dilution of 1:5,000. Then we incubated with secondary antibody. We used the ImmPRESS VR anti-mouse IgG HRP Polymer Detection Kit (Vector Labs) and this stage of the immunostaining procedure was completed by reacting the sections for approximately 30 minutes in Vina Green chromogen 5 substrate (Biocare Medical). Counterstaining was performed with hematoxylin QS (Vector Labs H-3404).

We tested various chromogen colors in all combinations for double staining, namely: Brown-ImmPact DAB Vector Laboratories SK-4105; Purple - ImmPact VIP Vector Laboratories SK-4605; Red/orange - Romulin AEC Chromogen Kit Biocare Medical RAEC810L; Green - Vina Green Chromogen Kit Biocare Medical BRR807AH; Black - HighDEF Black IHC Enzo ADI-950-171-0030; Yellow - HighDEF Yellow IHC Enzo ADI-950-170-0030. As control, we performed RNS immunoincubation sequence with buffer vehicle in the first stage instead of anti ChAT antibody and with buffer vehicle in the second stage instead anti MBP antibody.

Images were acquired by a Zeiss AxioLab A1 microscope using digital color cameras Zeiss ERc5s and Zeiss AxioCam 305, controlled by the Zeiss Zen Blue software. Image processing was accomplished using Fiji software (9).

3. Results

Of the various chromogen ‘colors’ tested in all combinations for double staining, DAB light brown appeared to be a robust reaction product color that withstood the second antigen retrieval step and the subsequent second immunostaining. Optically, RNS provides good contrast between the light brown of the choline acetyltransferase reaction product and the green of the myelin basic protein reaction product (figure 1). Contrast in double stained sections of the ChAT reaction product was as good as in single stained sections (figure 2). Nuclear morphology and location are clearly identified by the counterstaining with hematoxylin.

Analysis of the image in the RGB system showed how the color associated with myelin has low values in the RED channel, while the color associated with ChAT has low values in the BLUE channel. The combination of the two channels provides a simple and automated way to highlight ChAT-positive and ChAT-negative myelinated fibers, thus opening the way for a possible quantification of the myelinated motor fiber component in the nerve (Figure 2).

When applied to the study of the intact nerve, RNS shows how myelinated ChAT-positive motor fibers have the typical fasciculation pattern, grouping together in bundles. Myelinated ChAT-negative fibers are clearly discriminated as well (Figure 1).

When applied to the study of the transected injured nerve, RNS shows an immediate efficacy in differentiating between the early stages (3 days post-injury) of retrograde degeneration in the proximal stump and Wallerian degeneration in the distal stump. A key feature for both is the enlarged diameter of degenerating axons: however, while ChAT is lost in nearly all the distal axonal stumps, it is retained in the majority of proximal axonal stumps. The use of

MBP staining alone does not pick up this difference so well, as shown in figure 2. RNS also shows how limited is the component of ChAT positive regenerating axons that enter the conduit after 4 months (figure 2).

4. Discussion

Our main interest is the study of nerve regeneration within artificial nerve-guides (“conduits”) which are used in the clinic to bridge nerve gap-lesions (10–12). The easy histological identification of myelinated motor fibers is important because motor recovery is often less complete and slower than sensory recovery (8). The factors and processes responsible for the slower recovery of motor fibers are still poorly understood. One possibility for this differential behavior may be the degeneration of the muscular motor end-plate, which may occur during the interval in which the nerve repairs. However, the evolution of motor fiber regeneration has been rarely investigated at the level of the stumps and during the very early phases of nerve regeneration, when the motor-plate is presumably less affected.

In our main study we closely monitor five different regions at the level of the gap lesion: 1- the transverse section of the proximal stump about 1 mm proximal to the lesion; 2- the transverse section of the regenerated tissue about 1 mm distal to the proximal stump; 3- the longitudinal section of the artificial nerve-guide; 4- the transverse section of the regenerated tissue about 1 mm proximal to the distal stump; 5- the transverse section of the distal stump about 1 mm distal to the lesion (11). Before developing RNS, we used to sequentially stain four consecutive slides for each section with: hematoxylin/eosin; Masson trichrome staining; single ChAT immunostaining; single MBP immunostaining. We, then, usually compared the pictures (as these were from adjacent sections, their overlap was usually incomplete) and in this way we acquired a documentation of the myelinated motor fibers recovery. This traditional approach can be considered lengthy and cumbersome in comparison with RNS. The significance of the results obtained by the RNS method is in its direct identification of myelinated motor fiber (and, reciprocally, non-motor fibers) in a single slide. With RNS there is a reduction in the number of slides per section and, consequently, in the time required for reading.

We are also aware that a major advantage of the RNS procedure is that retrospective studies can be conducted on material stored for some time in the Pathology Department’s archives. Verification of the efficacy of RNS on archived material and comparison with fresh material (as we are presently using) will require a major study that we will enterprise in cooperation with neuropathologists. As a further future development, we are now studying the possible incorporation of a specific marker for sensory fibers in the protocol and, at the same time, we are applying RNS to the study of spinal cord regeneration promoted by artificial devices. The latter is a new field of research where the sequential immunostaining of ChAT and MBP on cryo-sections (13) has been applied already: we think that it could be associated with, or replaced by, our RNS method.

5. Conclusions

The routine combination of choline acetyltransferase (ChAT) and myelin basic protein (MBP) immunostaining provides a highly specific, highly contrasted paraffin-embedded sections where the optical differentiation of myelinated motor fibers is easy and straightforward. This method will likely simplify and speed-up the routine histological study of nerve regeneration and will contribute a better identification of the nerve motor component.

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Highlights

- We combined immunostaining of ChAT and MBP on a single paraffin embedded section.
- Cholinergic myelinated fibers were easily identified.
- This method will simplify and speed-up the histological study of nerve regeneration

Figure 1.

(A) RNS provides a good contrast between the green of myelin basic protein and the light brown of the choline acetyltransferase. Nuclei are identified by the counterstaining with hematoxylin. Myelinated ChAT-positive motor fibers (brown open-arrows) show the typical fasciculation pattern, grouping together in bundles. Large myelinated ChAT-negative fibers (white solid-arrow) are clearly discriminated (63X; scale bar 50 micron). The top-right and bottom-right insets show the single-ChAT (B) and single-MBP (C) immunostaining respectively, on sequential sections. Despite they provide the same information as in RNS, we found that their combined reading is not intuitive (40X; scale bar 50 micron).

Figure 2.

(A) Splitting the RGB channels of a RNS image (RNS) shows that the myelin signal has low values in the (B) RED channel (RED), while ChAT signal has low values in the (C) BLUE channel (BLUE). The combination of the two channels (D) provides a simple and automated way to highlight ChAT-positive and ChAT-negative myelinated fibers (RB-PRO). As control, RNS (ChAT/MBP) (E) was compared with an immunoincubation sequence: with buffer vehicle in the second stage instead anti MBP antibody (ChAT/no) (F); with buffer vehicle in the first stage instead of anti ChAT antibody (no/MBP) (G); with no antibodies (no/no) (H). Comparison of the ChAT/MBP to ChAT/no shows how contrast in double stained sections of the ChAT reaction product was as good as in single stained sections (20X; scale bar 100 micron). When applied to the study of the transected injured nerve 3 days post-injury, RNS shows a clear differentiation between retrograde degeneration in the proximal stump (Prox RNS) (I) and Wallerian degeneration in the distal stump (Dist RNS) (J) and evidences how ChAT is retained in the majority of proximal axonal stump. The use of MBP staining alone, on the opposite, produces pictures that may suggest a kind of similarity (Prox MBP (K); Dist MBP (L)) (20X; scale bar 100 micron). In the regenerated nerve that enters the conduit after 4 months (Regenerate) (M), RNS shows how a limited number of ChAT faintly positive regenerating axons (black arrows) entering the conduit after 4 months, in comparison with numerous new myelinated axons of smaller diameter (dashed circles) (40X; scale bar 50 micron).