Reciprocal Nerve Staining (RNS) Allows the Concurrent Detection of Myelin Basic Protein and Choline Acetyltransferase on Paraffin-embedded Sections

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Introduction. Myelin Basic Protein (MBP) immunostaining is a well established methodology to differentiate between myelinated and not myelinated fibers. Choline acetyltransferase immunostaining can differentiate between motor and non-motor (largely sensory) fibers. These methodologies, largely employed in cryosection and fluorescence microscopy, have been recently individually applied to paraffin-embedded sections. Traditional paraffin embedded sections present the advantage to have a relatively simple methodology and allow decades long storage life. They can be easily shared among laboratories worldwide. In the study of nerve regeneration, a clear-cut differentiation between motor and sensory fibers is critical; at the same time, myelinated fibers must be clearly identified. We developed a histological protocol where both MBP and ChAT are stained together with sufficient contrast on paraffin-embedded sections. The technique has been called reciprocal nerve staining (RNS).

Materials and Methods. The sections were deparaffinized, hydrated with alcohol, and subjected to heat-induced epitope retrieval with citrate buffer, pH 6.0 for 20 minutes at 98 degrees using. The sections were blocked with 10% normal donkey serum for 30 minutes followed by 48-hour incubation of sheep polyclonal to choline acetyltransferase antibody (Abcam 18736) at dilution of 1:150. The secondary antibody donkey anti-sheep polymer was used followed by 5 minutes of DAB chromogen substrate (Vector Labs SK-4105). After completion of 1st primary antibody (ChAT), slides were again subjected to heat-induced epitope retrieval with citrate buffer, pH 6.0 for 20 minutes at 98 degrees. The sections were blocked with 10% normal horse serum for 30 minutes followed by 1 hour incubation of mouse monoclonal anti-myelin basic protein antibody (Abcam 62631) at a dilution of 1:5,000. The secondary antibody ImmPRESS VR anti-mouse IgG HRP Polymer Detection Kit was used (Vector Labs) followed by 30 minutes of Vina Green chromogen substrate (Biocare Medical). Counterstaining was performed with hematoxylin QS (Vector Labs H-3404).

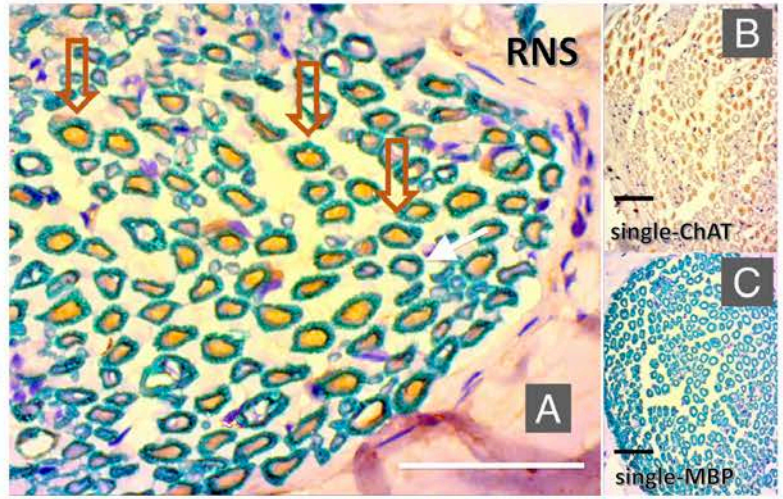
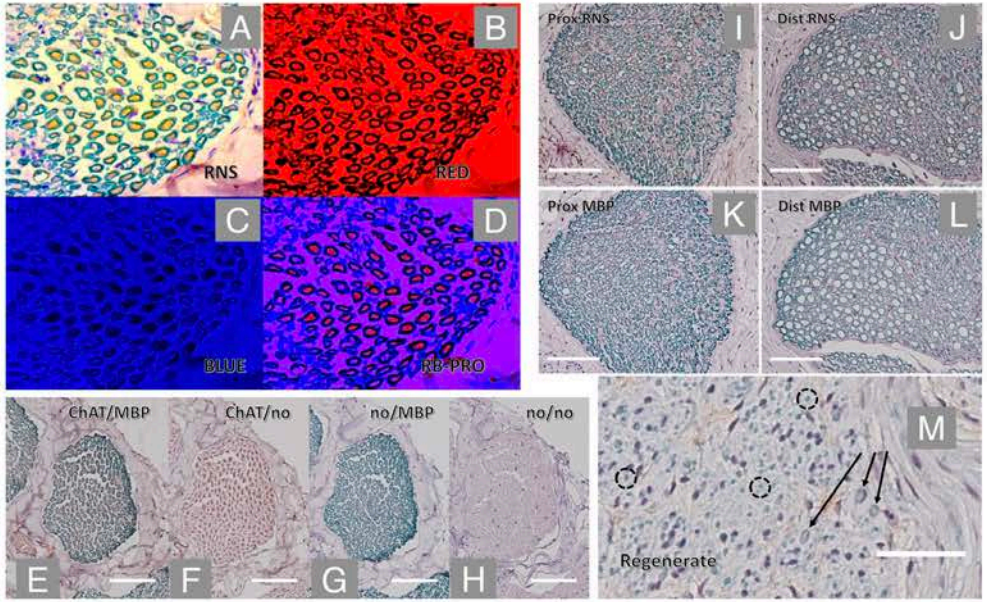


Figure 1 ->

(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).



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.

<- 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).

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.

Reference: Merolli A, Louro P, Kohn J. Reciprocal nerve staining (RNS) for the concurrent detection of choline acetyltransferase and myelin basic protein on paraffin-embedded sections**. J Neurosci Methods**. 2019 Jan 1;311:235-238

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