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A modified technique for high-resolution staining of myelin

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Abstract

This report describes a new modification of the Gallyas method for staining myelin in fixed brain tissue and compares results of multiple myelin-visualizing techniques in normal common marmoset (*Callithrix jacchus*), normal macaque monkey (*Macaca mulatta*), and a human with multiple sclerosis. The new modification involves immersion in 10% formalin following impregnation in ammoniacal silver nitrate, and the use of a low concentration of 4% paraformaldehyde in the developer. This improved technique is less sensitive to post-mortem tissue handling, temperature, and minor contaminants, allowing a more straightforward implementation in the laboratory setting. It permits simple user-controlled development of the reaction product to maximize contrast in the area of interest, resulting in high contrast staining not only of large axonal bundles, but also thin fascicles throughout tissue sections.

Myelin staining in visual cortex of an Old World monkey and a New World monkey reveals similar patterns in the new myelin silver stain, the Gallyas stain, and myelin basic protein immunohistochemistry. The most heavily myelinated areas occupy the edges of blobs, but neither the most lightly stained nor the most darkly stained areas are always in our outside a blob. This indicates a more complex pattern between myelinated axons and blobs than previously suggested. While the new myelin silver stain, darkfield microscopy, the Luxol Fast Blue stain, the Gallyas stain, and myelin basic protein immunohistochemistry all permit visualization of myelin in the CNS, each technique has its own merits and pitfalls; careful evaluation of individual study requirements would best determine which methods are the most useful.

Keywords: Gallyas; Silver; Cerebral cortex; Marmoset; Macaque; Demyelination; Auditory cortex; Medial geniculate; Primate; Multiple sclerosis; Luxol Fast Blue; Darkfield; Myelin basic protein; Visual cortex; Blobs

1. Introduction

Several disciplines frequently require the visualization of myelin in post-mortem brain tissue. One example is neuropathology and the study of diseases that cause the degeneration of myelin, such as multiple sclerosis. Neuroscientists examining the function or organization of brain regions often find that variations in myelination can be used to distinguish areas anatomically.

Various techniques have been developed to visualize myelinated axons in the central nervous system, depending on the nature of the study. In wet, unstained sections, myelination can be seen and photographed using a light microscope with regular illumination (Richter and Warner, 1974) or with darkfield illumination (Horton and Hocking, 1997). Basic staining techniques such as the Weil stain (Weil, 1928; Berube et al., 1965), the Pal-Weigert Method (Weigert, 1884,1885; Clark and Ward, 1934), Luxol Fast Blue (Klüver and Barrera, 1953), and immunocytochemical localization of myelin basic protein (Horton and Hocking, 1997) can be used when high resolution of fibers is not required. When a detailed stain including very small myelinated fibers is required, however, many investigators use the silver staining technique first developed by Gallyas (1979), which is based on the binding of colloidal silver to myelin for viewing by light microscopy.

The Gallyas stain for myelin has proven capricious, as it gives variable results depending on the post-mortem handling of the tissue, the ambient temperature during physical development, the quality of reagents used, and the proficiency of the experimenter performing the technique. Because of these limitations, several techniques have been developed for staining myelin both rapidly and with ease. Such benefits have come at the expense of high-contrast and detailed staining of fine fascicles. Yet, the Gallyas method remains the standard used by many

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neuroanatomists and physiologists due to the quality of data it can produce, and its wide application for staining either frozen or embedded sections of variable thickness (5–100 μm).

Two issues with the silver stain for myelin are the difficulty in controlling the level and speed of staining, and the unpredictability of background levels from section to section. A chemical method to attenuate silver staining has been used to increase contrast and for general de-staining. In cytological preparations, Meywald et al. (1995) found that the use of a silver reducing agent, potassium ferricyanide, after the staining procedure was the best method for obtaining a high signal-to-noise ratio, in addition to allowing for complete de-staining, if necessary. The use of this "bleaching" step after development serves two purposes for the myelin staining procedure we describe; one is to de-stain tissue which has been developed too far, the other is to increase contrast in the staining pattern between myelin and background. Indeed, others have found this reducing agent helpful in obtaining the best contrast in silver staining for myelin in cerebral cortex (Jain et al., 1998).

We have developed a modification of the Gallyas silver stain, which offers higher reproducibility and greater control over development, while being less sensitive to error and contamination. Two critical steps are added to the standard Gallyas method: (1) the introduction of a fixation step, using 10% formalin, between impregnation and development helps to soften folds in tissue that may have been introduced in the lipid solvent step, opening the tissue to more even staining during physical development, and providing a safe stopping point mid-stain, making possible the short-term storage of half-processed sections. (2) The use of a ferricyanide bleaching step after developing increases the signal-to-noise and enables the tissue to be bleached out completely and re-developed if the bleaching is excessive or if staining is not at the desired level.

2. Materials and methods

Seven common marmosets (*Callithrix jacchus*), at the end of electrophysiological studies, were sedated with ketamine (IM) and euthanized with an IP lethal dose of pentobarbital sodium/phenytoin sodium (Euthasol Euthanasia Solution, Virbac) and perfused transcardially with heparinized phosphate buffer (pH ∼7.0) followed by 4% paraformaldehyde (EM Grade, Ted Pella #18501) in 0.1 M phosphate buffer (pH ∼7.0). The brains were immediately removed and cryoprotected in 20% sucrose (in 0.1 M phosphate buffer) for ∼24–48 h, after which they were blocked and frozen at −80 °C. Most of the blocked tissue was cut at 30 μm, perpendicular to the lateral sulcus, on a freezing microtome. The parietal lobe (to include all of V1 and V2) from three animals was thawed, flattened so that the pial surface was facing upward and parallel to the microtome blade, re-frozen using dry ice, and cut at 30 μm. Some sections were mounted onto subbed slides and stained for Nissl, or stained for cytochrome oxidase (CO). Wet, unstained flattened sections designated for staining with one of the myelin techniques were viewed under darkfield illumination and digitally imaged. Sections designated for the Gallyas or new myelin stain were placed into 10% formalin (J.T. Baker #2106-01) for a variable time from 4 weeks to greater than 8 months. Remaining sections were stained for other myelin techniques as outlined below.

Visual cortex tissue from one visually-normal macaque (*Macaca mulatta*), used in anatomical studies of extrastriate cortex, was also processed to compare with results from marmoset. The animal was sedated with ketamine, euthanized with an IV injection of pentobarbital sodium (100 mg/kg), and perfused transcardially with 0.1 M phosphate buffer followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brain was removed, blocked, and cryoprotected in 20% sucrose for several days. A block of primary visual cortex was flattened, frozen, and sectioned at 30 μm on a freezing microtome. Adjacent sections were processed for CO, the new myelin silver stain, and myelin basic protein immunohistochemistry using identical methods as those in the sections from marmoset.

A sample of human tissue from a multiple sclerosis (MS) case was obtained from the Pathology Department of the Johns Hopkins School of Medicine. Post-mortem time and handling of tissue were not known, except that the tissue block had been preserved in formalin at room temperature for at least several months. The block was cryoprotected in 20% sucrose until it sank and then frozen just prior to sectioning. Sections were cut on a freezing microtome at 40 μm, rinsed in phosphate buffer, and then placed in 10% formalin for 7 days before being processed for the new myelin silver stain.

2.1. Processing for the new myelin silver stain

2.1.1. Pre-treatment with a lipid solvent

Floating sections were removed from 10% formalin and placed in 2:1 pyridine: acetic anhydride (pyridine-Fisher #P368, acetic anhydride-Fisher #A10) for 30 min. Sections were then re-hydrated slowly by changing through washes of 80, 60, 40, and 20% pyridine, followed by two water rinses. Floating sections were kept in a large glass Petri dish, with washes pipetted off, instead of transferring sections, to maintain tissue integrity.

2.1.2. Impregnation with colloidal silver

Sections were immersed in fresh ammonical silver nitrate solution (see solution ingredients below) for 45 min. Excess (unbound) silver was removed by washing sections in three changes of 0.5% acetic acid (total time approximately 10 min).

Ammoniacal silver nitrate:
100 mL ddH_2O
100 mg ammonium nitrate (Sigma Ultra #A7455)
100 mg silver nitrate (Sigma Ultra #S-8157)
0.3 mL 4% NaOH solution

2.1.3. Re-immersion in fixative

Following the final wash in 0.5% acetic acid, sections were transferred into freshly prepared 10% formalin and were stored until the second part of the staining procedure began. The time of this fixation step varied from one day to several months, depending on the needs of the experimenters.

2.1.4. Developing bound silver particles

The sections were removed from 10% formalin and washed twice in 0.5% acetic acid. During the washes, the developer was made fresh from equal volumes of two stock solutions of (1) 5 g sodium carbonate (anhydrous, Sigma #S6139) in 100 mL ddH_2O; and (2) 0.2g ammonium nitrate (Sigma Ultra #A-7455), 0.2g silver nitrate (Sigma Ultra #S-8157), and 1.0 g tungstosililic acid (hydrate, Sigma #T-2786) in 100 mL ddH_2O; and 73 μL of 4% paraformaldehyde was added (Ted Pella #18501) per 20 mL total developer volume. The sections were immersed in the developer solution for a time ranging from 10 min to over 1 h, depending on the speed of reaction. The reaction was monitored under a dissecting microscope and stopped by immersing in two washes of 1.0% acetic acid when the experimenter was satisfied with the level of detail in the staining (e.g. when small fascicles are visible in cortex).

2.1.5. Bleaching to de-stain overdeveloped sections and/or increase contrast

After washing sections in acetic acid, they were immersed in 0.1% potassium ferricyanide (Fisher #P232) until the desired level of differentiation was reached. They were then washed in a solution of 0.5% sodium thiosulfate (anhydrous, Fisher #S446) for 1 min, followed by three washes in water. Sections were immediately sorted and mounted, onto subbed slides, out of warmed subbing solution (2 g gelatin, 200 mL ddH_2O, 100 mL 70% EtOH, and 100 mL 95% EtOH).

2.2. Modified Gallyas, Luxol Fast Blue, and myelin basic protein controls

Three hemispheres from two different marmosets were processed using a slightly modified (addition of a bleaching step) Gallyas stain. The use of the modified Gallyas stain, as opposed to following the exact protocol published by Gallyas, was necessary because the tissue developed uniformly and halting this process at any point would not result in discernable myelin. Once the sections were "overdeveloped," the bleaching step de-stained the unmyelinated areas more effectively than myelinated areas, making differentiation possible. This modified Gallyas stain differed from the method presented in this paper in several key ways. First, there was no fixing step between impregnation and development. Second, the developers were mixed from three stock solutions according to ambient temperature, as in the original Gallyas paper (1979). Finally, the sections were overdeveloped for 10 min and then de-stained in the ferricyanide bleaching process.

Three hemispheres in three different animals were processed for Luxol Fast Blue staining. The following protocol was used:

1. Mount fresh tissue and dry slides on heater at least 2 days.
2. Drop into stain overnight.
3. Rinse (briefly) in 70% EtOH.
4. Put into 0.05% Li_2CO_3 for 10 s.
5. Rinse in 70% EtOH.
6. Repeat as necessary until differentiated (view under scope).
7. Counterstain at this time, if desired.
8. Dehydrate, clear, and coverslip.

Luxol solutions	
A.	Stain—allow to age for at least 1 month: 0.1 g Solvent Blue 38 (powder), 100 mL 96% EtOH (192 mL EtOH + 8 mL ddH_2O), 0.5 mL 10% Acetic Acid (1 mL glacial acetic acid + 9 mL ddH_2O);
B.	Li_2CO_3 Solution—0.1 g/200 mL ddH_2O.

Sections in four hemispheres of three different marmosets and one hemisphere from macaque were stained for myelin basic protein using immunohistochemistry. After being cut, sections were placed in ethanol/hydrogen peroxide solution for 15–20 min to remove any remaining red blood cells. Sections were then blocked in 3% normal horse serum for 2 h, then placed in a 1:800 dilution of primary antibody [mouse anti-myelin basic protein (MBP) (129–138), Chemicon #MAB382] for 24–48 h. After successive incubations in biotinylated secondary antibody and an avidin–biotin binding complex (Vectastain ABC standard peroxidase kit, Vector Laboratories), the bound complex was visualized using diaminobenzidine and hydrogen peroxide. Sections were mounted onto subbed slides, dried, and the stain was amplified using osmium and thiocarbohydrazide.

3. Results

Fig. 1 shows a series of rostral-to-caudal sections in one marmoset to illustrate the consistency of staining and quality of signal-to-noise using the new modification. The spacing between sections is not regular and is intended to show highlights of key areas such as the globus pallidus, lateral and medial geniculate, hippocampus, and areas of cerebral cortex (the primary sensory and motor areas are clearly distinguished from adjacent secondary areas by darker staining).

Several higher-magnification photomicrographs of various areas are shown in Figs. 2–4. For studies of cerebral cortex, the present modification provides a high level of fiber detail. Primary auditory cortex can be easily differentiated from adjacent sensory areas (Fig. 2) by the heavier myelination present, as is also the case in other primary sensory areas. Arrows indicate the border between primary auditory cortex (A1) and belt areas (medial, MB; and lateral, LB). Heavy myelination in A1 begins in Layer 3 and continues into Layers 4–6. This myelination pattern is not seen in auditory association areas, such as medial or lateral belt. Thalamic nuclei (lateral geniculate and medial geniculate) can be seen in Fig. 3A. Ventral, medial, and dorsal subdivisions of the auditory thalamus, or medial geniculate nucleus (MGN), are distinguishable based on fiber patterns (Fig. 3B). Images of the inferior colliculus, seen in Fig. 3C, show a higher level of fiber detail than is seen in most myelin stains, allowing for straightforward delineation of subdivisions.

Fig. 4 shows photomicrographs of human MS tissue. A low magnification photomicrograph (top left) demonstrates the quality of the stain over the entire section, despite the poor condition

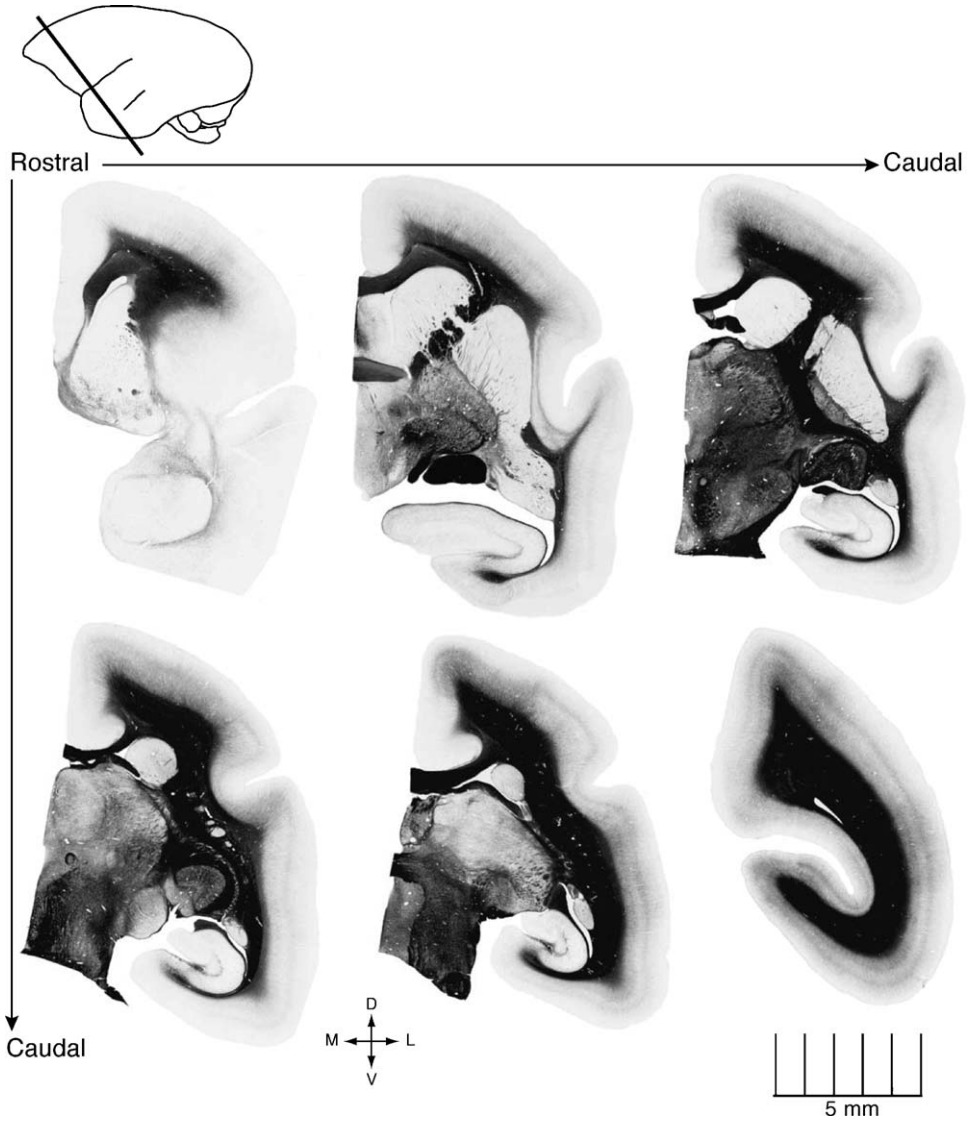

Fig. 1. Irregularly-spaced sections, processed using the new myelin silver stain, through the brain of one marmoset are shown. Sections are ordered rostral-to-caudal, left to right and top to bottom, with the top left section being the farthest rostral and the bottom right the farthest caudal. Top left: line rendition of one hemisphere of a marmoset brain with a thick black line indicating the plane of sectioning perpendicular to the lateral sulcus. Bottom center: orientation. Bottom right: scale bar.

of the tissue (part of cortex was torn during processing, even though it was handled gently). Note the lesion caused by MS at the bottom of the section, where there is a complete degeneration of myelin and little or no staining. Medium and high magnification photomicrographs of cerebral cortex are shown at the bottom (left and right). Overall, this tissue sample does not have the extreme fiber detail and contrast seen in the marmoset tissue, most likely due to post-mortem factors, but the modified procedure we used shows more detail than Luxol Fast Blue or Weil's. Blood vessels containing RBCs are also visible in these sections, but are clearly distinguishable from myelinated fibers by size, shape, and orientation. At medium magnification (right, top), the boundary of the unstained MS lesion and the deeply stained myelin stands out as a sharp border. A higher magnification photomicrograph (right, center) further demonstrates the remarkable perimeter of degeneration, in a consistent pattern regardless of fiber tract orientation.

3.1. Method comparison

Photomicrographs of tissue processed with the previously-used Gallyas modification are shown in Fig. 5A. Typical problems, which are difficult to control for are illustrated in this figure. Inconsistent staining within a section is evident (top), where circled areas have increased background staining not related to myelinated fibers. This background staining, which typically ranges from yellowish to brown to black, extends throughout all layers of cortex, and is clearly not fibrous in nature. Uneven staining which results from tissue folding during the staining procedure is also shown (bottom). Folding typically results in a sharp line of increased background staining, often very dark brown to black, obscuring any detail of myelinated fibers. The intense background seen in and around a folded area cannot be bleached out with ferricyanide while still maintaining proper staining of myelinated fibers.

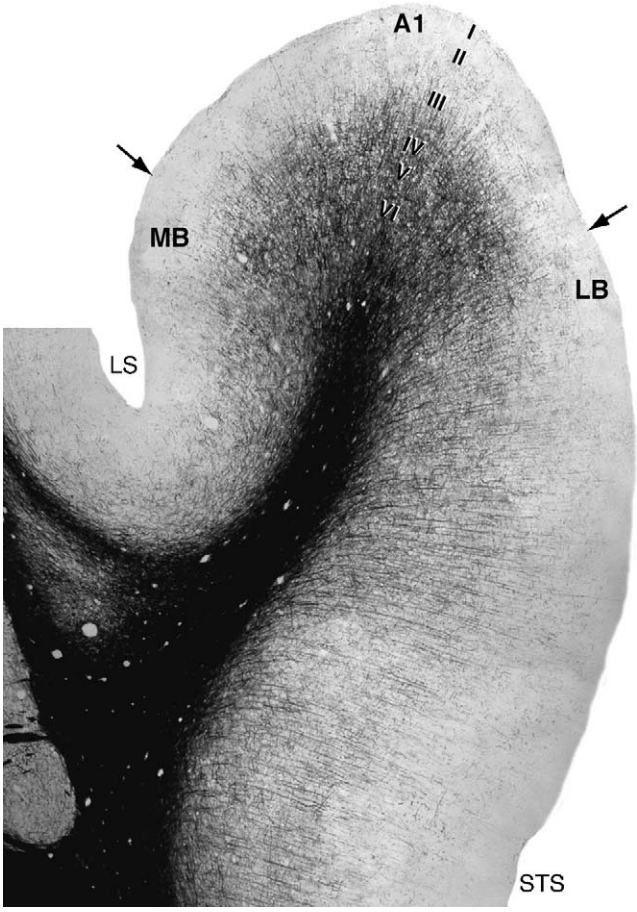

Fig. 2. One section through the auditory cortex of a marmoset, processed using the new myelin silver stain, is shown. Arrows indicate the border between auditory core (A1) and belt areas, medially and laterally. I–VI refer to cortical layer numbers. A1: primary auditory cortex, LB: lateral belt, MB: medial belt, LS: lateral sulcus, STS: superior temporal sulcus.

For comparison to the Gallyas method, sections processed for Luxol Fast Blue can be seen in Fig. 5B. This method is sufficient to mark the approximate border between primary auditory cortex and secondary areas (arrow), but the lack of resolution down to the level of individual fibers, makes a precise border difficult to detect. The general properties of this stain, which illustrates the extent of myelination by differences in staining intensity, are similar in resolution to those of the Weil and Weigert staining methods.

Fig. 5C compares staining for the myelin basic protein antibody with the new myelin silver stain presented in this paper. In areas sparse of myelinated fibers, such as the Islands of Calleja (Nissl staining is shown for visualization of cell bodies, top left), the myelin basic protein stain (MBP, top right) shows very good resolution of fibers, including areas not stained using the new silver technique (NM, top center). However, the new technique (bottom left) is far superior to visualize myelinated fibers throughout all layers of cerebral cortex and into white matter, whereas the stain for myelin basic protein (bottom right) is uniform and loses its power of resolution in myelin-dense areas, such as cortical layers 3–6 and white matter.

Adjacent sections (top to bottom) in visual cortex from one animal are shown in Fig. 6. Cytochrome oxidase stains are shown (top and bottom) for comparison of lamination and extent of white matter. To the left of stains for myelin basic protein, Gallyas, and the new technique presented in this paper are darkfield photomicrographs from the same sections taken prior to staining. The myelin basic protein stain appears uniform despite the lamination apparent in the darkfield image. Despite low contrast of fibers against the background level, cortical lamina and myelin-dense areas are readily apparent in the Gallyas stain. In comparison, the new staining technique shows an extremely high resolution between fibers and background, and lamina and white matter are clearly distinguishable.

3.2. Complex patterns of myelination in flattened V1 of marmoset and macaque

A semi-regular pattern of myelination was seen in Layers 2 and 3 of marmoset flattened visual cortex in both the Gallyas and new myelin staining techniques. The pattern consisted mostly of medium-tone patches, fewer dark patches, and sparse light patches. To determine if these patterns coincide with CO patches (blobs) seen in the same area, we first traced the lightest (presumably the most myelin-sparse) areas, then lined up the trace on an adjacent section stained for CO using blood vessels as land marks. Results from one animal are shown in Fig. 7. Photomicrographs of the sections used (Gallyas, in this example, and CO) are shown at the top, and an enlarged view of the CO-stained section is shown at the bottom with white outlines tracing the lightest areas from the Gallyas-stained section. The areas stained lightly for myelin appear most commonly in inter-blob space but do occasionally coincide with a blob. These details are suggestive of a complex relationship between staining patterns.

A comparison with the findings in marmoset was performed using the new myelin silver staining technique and immunohistochemistry for myelin basic protein in sections of macaque V1. Fig. 8 shows a section stained for CO with an overlay of the most lightly-myelinated areas, based on the new myelin stain, using the same technique as in Fig. 7. As in the marmoset, the areas of sparse myelination in macaque V1 fall largely outside of blobs. To explore whether densely myelinated areas correspond to blob patterns, photomicrographs of sections stained for myelin (new myelin and myelin basic protein) were contrast-enhanced, and then thresholded, to emphasize only the medium- to richly-stained areas. These areas were highlighted in white and lined up over photomicrographs of adjacent CO sections using blood vessels as land marks, as seen in Fig. 8. The dominant pattern is one in which the most heavily-myelinated areas are adjacent to blobs and frequently surround them. Rarely do these areas line up with the centers of blobs.

3.3. Optimized protocol

For most reliable results, use acid-cleaned bottles for solutions containing silver nitrate. Work with floating sections in a large glass Petri dish.

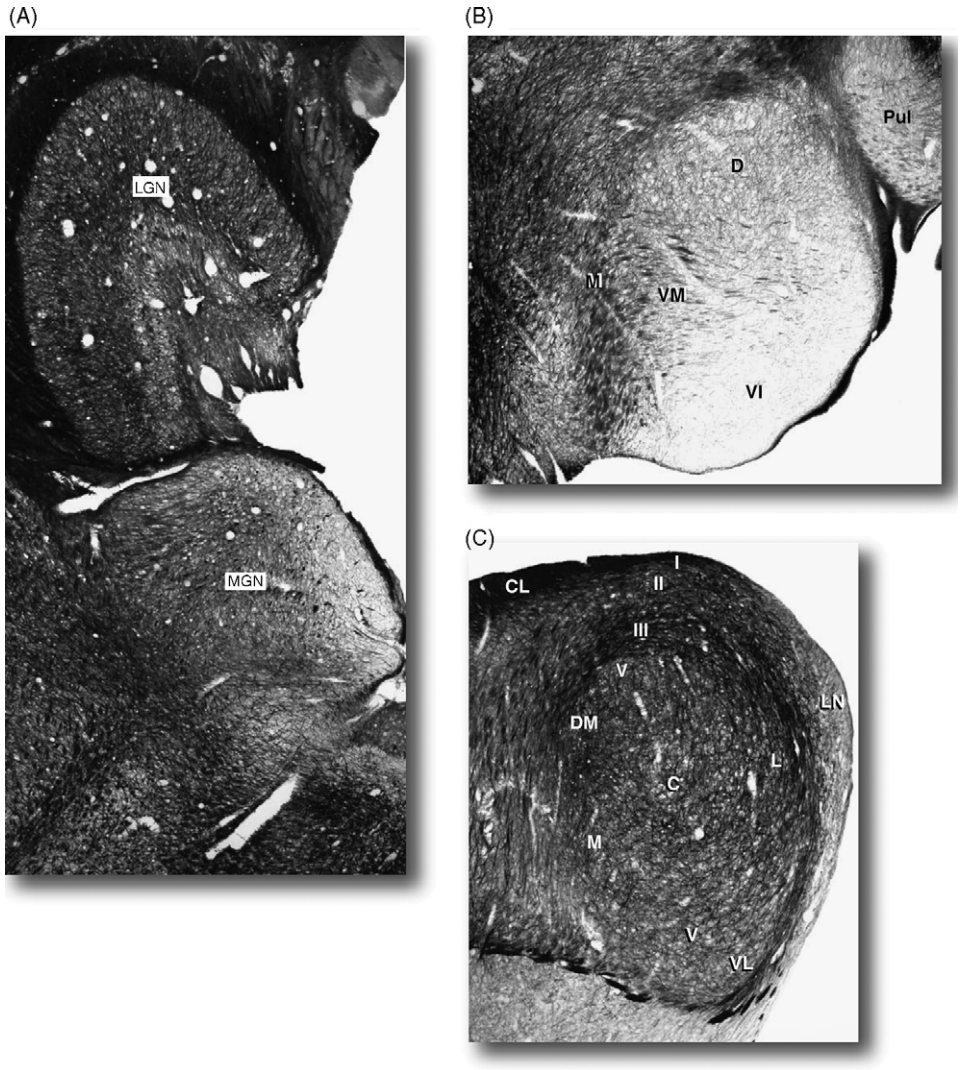

Fig. 3. A low-magnification photomicrograph of one section from marmoset processed for the new myelin silver stain through part of the thalamus (A), with higher-magnification detailed views of auditory thalamus (MGN, B) and auditory brainstem (inferior colliculus, IC, C) are shown. In (A), LGN: lateral geniculate nucleus, MGN: medial geniculate nucleus. In (B), the MGN, subdivisions are D: dorsal nucleus, M: medial nucleus, VM: medial part of the ventral nucleus, Vl: lateral part of the ventral nucleus (Pul: pulvinar, outside of MGB). In (C), the IC, subdivisions are CL: lateral commissural nucleus; dorsal cortex: Layers I, II, III, and IV; central nucleus: M, medial; C, central; L, lateral; paracentral nuclei: DM, dorsomedial; VL, ventrolateral; LN, lateral.

1. Fix sections in 10% formalin for 24 h–3 months.
2. Immerse sections in 2:1 pyridine: acetic anhydride for 30 min.
3. Do transfers/washes in 80, 60, 40, 20% pyridine, then 2 ddH_2O rinses (*Note*: sections will virtually disappear in this step. Work over a black surface under medium light for better detection, so you do not pipet sections).
4. Place in ammoniacal silver nitrate solution 45 min.
5. Wash for a total of 10 min with 3 changes of 0.5% acetic acid.
6. Transfer sections back into the 10% formalin, wrap container in Parafilm, and let sit for several days (can be more, up to several months).
7. Remove sections from formalin and wash 2× in 0.5% acetic acid before developing.
8. Mix equal volumes of developer solutions A and B. Slowly and carefully add 73 μl of 4% paraformaldehyde/20 mL of developer, either while stirring continuously at high rate, or shaking vigorously. If solution turns white or grey, you need to start over.
9. Develop sections in developer from Step 8. This process can range from ∼15 min to +1 h, depending on how long they were in fix from Step 6. Watch the development under a dissecting microscope and quickly move to Step 10 when they have reached the preferred level of staining. If they overdevelop or have a higher background than desired, this can be taken care of with Step 11 (after completing Step 10).
10. Wash 2× in 1% acetic acid, 5 min each.
11. Optional step (for overdeveloped sections or increasing contrast): immerse in 0.1% potassium ferricyanide. Bleach until the myelin has differentiated sufficiently, monitoring the sections with the dissecting scope (sections can stay in the Petri dishes). This will likely be a very fast process unless

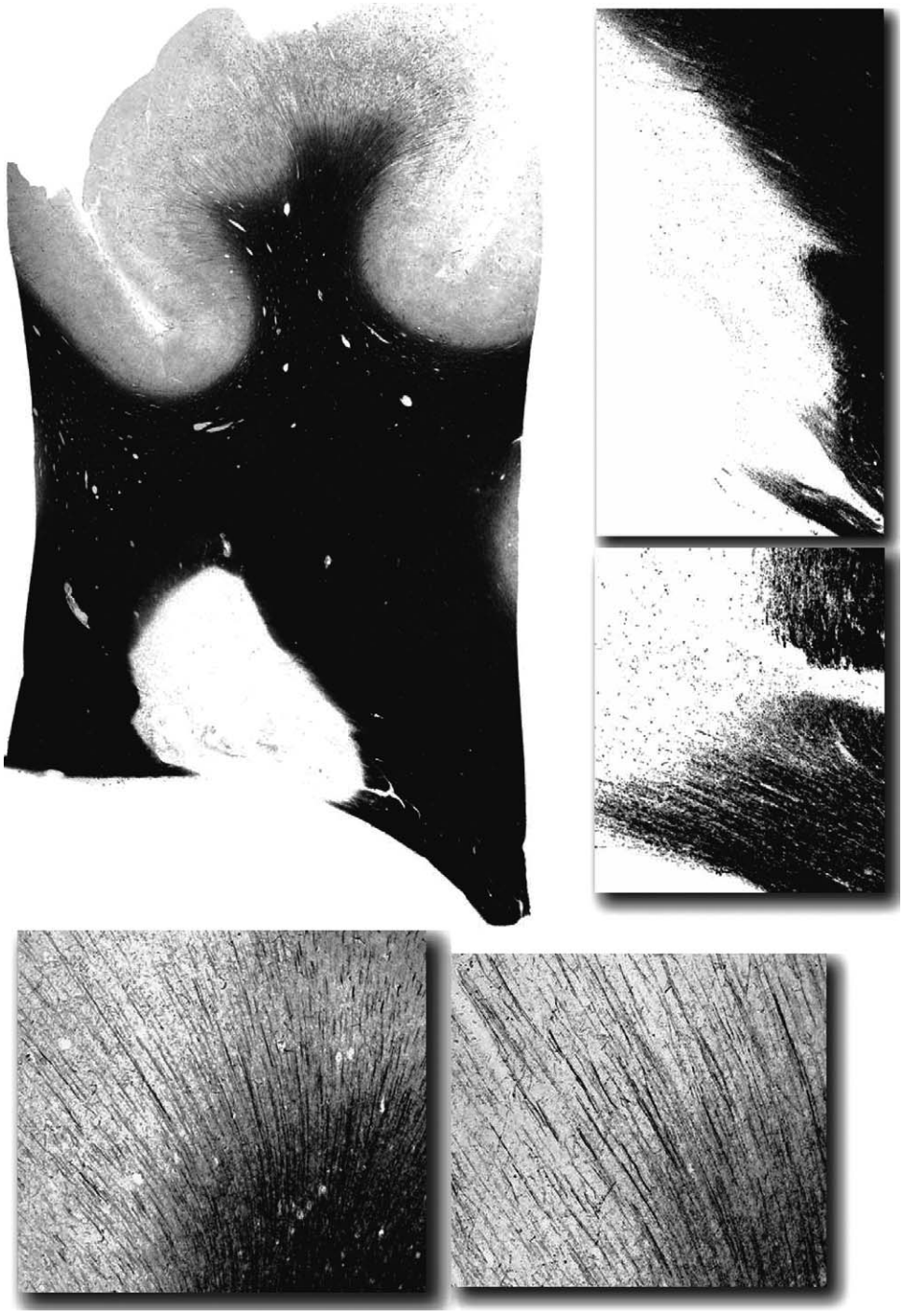

Fig. 4. Examples from a human MS case processed for the new myelin silver stain. Top left: low magnification view of a whole section. Right top and center: the MS lesion edge at medium magnification (5×, top) and higher magnification (10×: center). Bottom: middle and deep layers in normal cortex stained for myelin with the new modification are shown at two magnifications (5×: left, 10×: right).

they were extremely overdeveloped. Be ready to quickly transfer them to the next solution (Step 12).

If you bleach too much, completely bleach and wash in 0.5% acetic acid 2 min, then redevelop in fresh developer. Repeat Step 10 (and 11 if necessary).

12. Immerse in 0.5% sodium thiosulfate 1 min.
13. Wash 3× in ddH_2O, 5 min each. Mount onto subbed slides out of warmed subbing solution.

3.3.1. Solutions

Make fresh-

pyridine:acetic anhydride 2:1 (make only the volume you need-does not store well);

ammoniacal silver nitrate: 100 mg ammonium sulfate, 100 mg silver nitrate in 100 mL ddH_2O. Slowly stir in 0.3 mL of 4% sodium hydroxide.

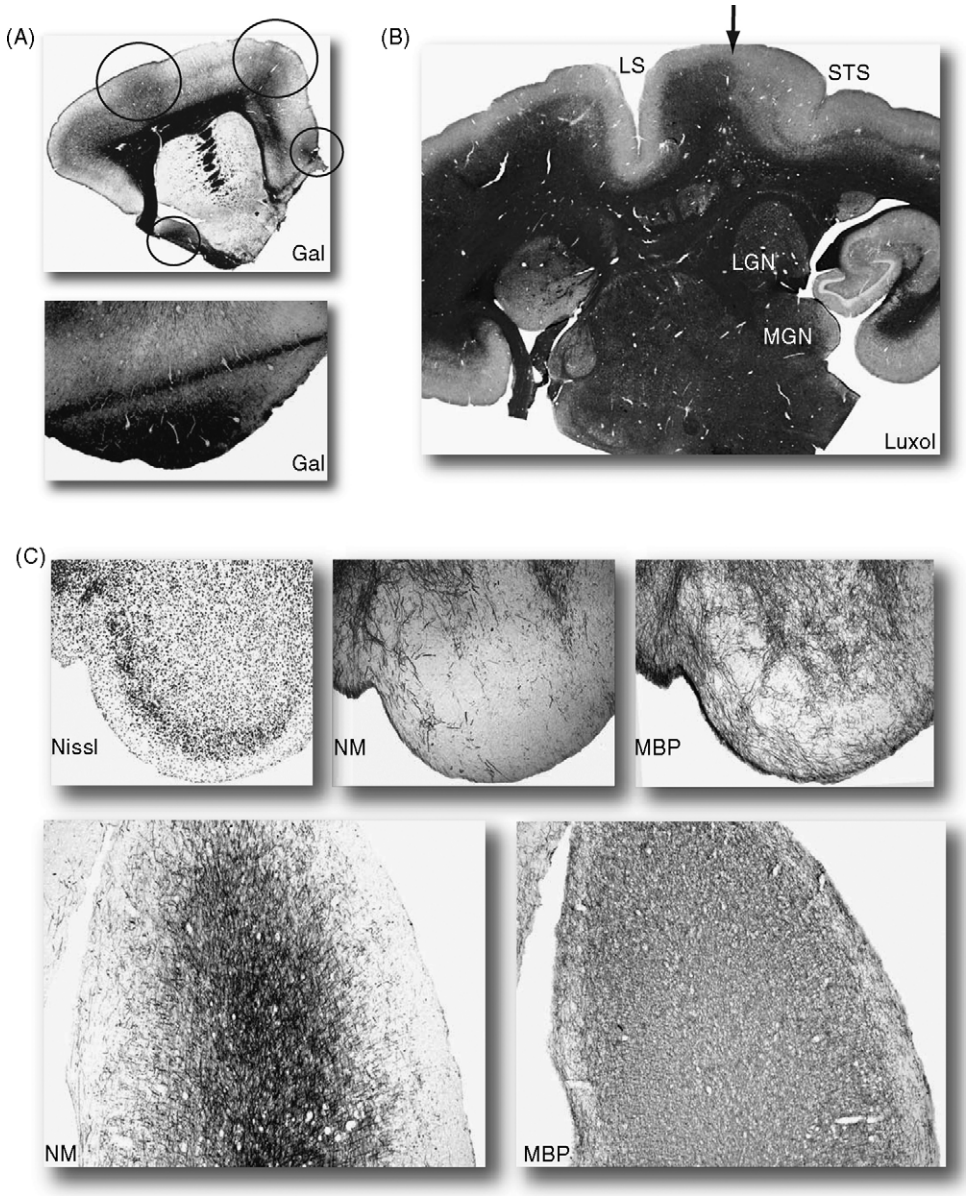

Fig. 5. Photomicrographs of sections of marmoset tissue processed with alternate methods for staining myelin. Undesirable results commonly seen using the Gallyas stain are shown in (A). Top: circles indicate areas of background staining unrelated to myelinated fibers, Bottom: example of staining around a fold introduced during the pyridine-to-water step. (B) Shows an example of one section stained for Luxol Fast Blue, with a color-dependent contrast enhancement to emphasize areas of myelin-specific staining from counter-stain in this photomicrograph (otherwise, a grayscale image would not show the true nature of the color difference visible under light microscopy). An arrow indicates the border between primary auditory cortex and lateral belt. Advantages and limitations of resolution down to individual fibers using myelin basic protein immunohistochemistry is shown in (C). Top row: adjacent sections through the Islands of Calleja stained for Nissl (right), the new myelin silver stain (NM, middle), and myelin basic protein (MBP, right). Using classical myelin stains and unstained sections as the standard for determining myelin distribution, this can be considered a sparsely-myelinated area. The rostral portion of temporal lobe containing part of the auditory core (R or RT) and secondary cortical areas is shown in adjacent sections stained for the new modification (NM, left) and myelin basic protein (MBP, right).

Developers can be stored:

A. Five grams of sodium carbonate (anhydrous)/100 mL ddH_2O;
B. 0.2 g ammonium nitrate, 0.2 g silver nitrate, 1.0 g tungstosililic acid/100 mL ddH_2O, dissolved in given order.

4. Discussion

The modification presented in this paper has several advantages over the original Gallyas stain and the previously-used modified Gallyas (as in Jain et al., 1998). Two major benefits come from the addition of the fixation step, half-way through the staining procedure. One is that folding introduced during the pyridine-to-water steps can be softened and opened, allowing better uniform reagent penetration during development. There is no longer a need to keep sections flat through this process; thus, more tissue can be developed at one time in a container. Secondly, this modification allows users a stopping point, which can be used for a variety of reasons. For instance, we conducted a study across multiple blocks of tissue, and were able to take

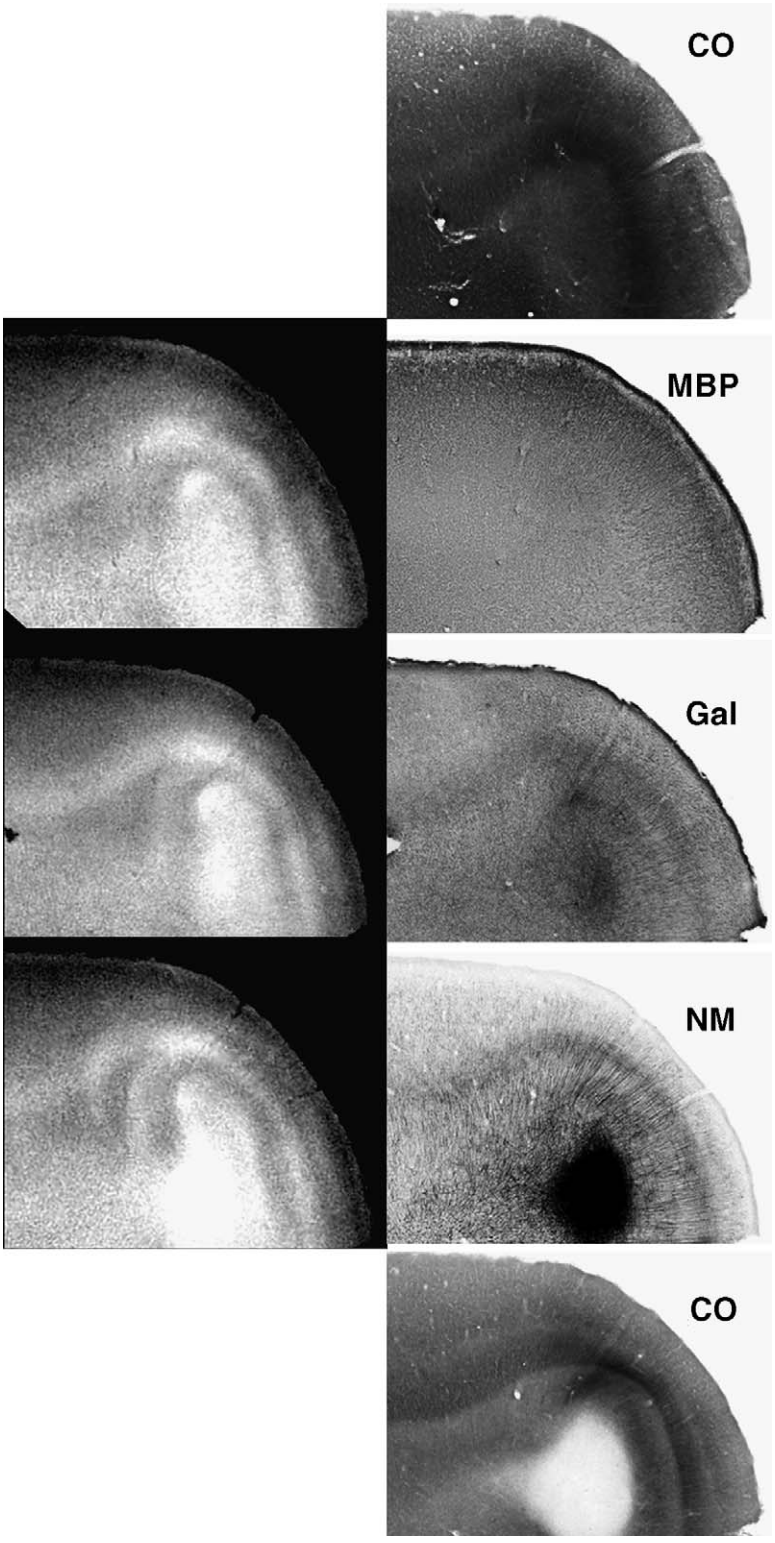

Fig. 6. Photomicrographs of adjacent sections through marmoset V1, in succession (from top to bottom) stained using three techniques: myelin basic protein immunohistochemistry (MBP), modified Gallyas (Gal), and the new myelin silver stain (NM), respectively. Corresponding unstained wet sections imaged in darkfield are shown to the left of each myelin stain. Cytochrome oxidase-stained (CO) sections are shown at the top and bottom to better visualize lamination and white matter.

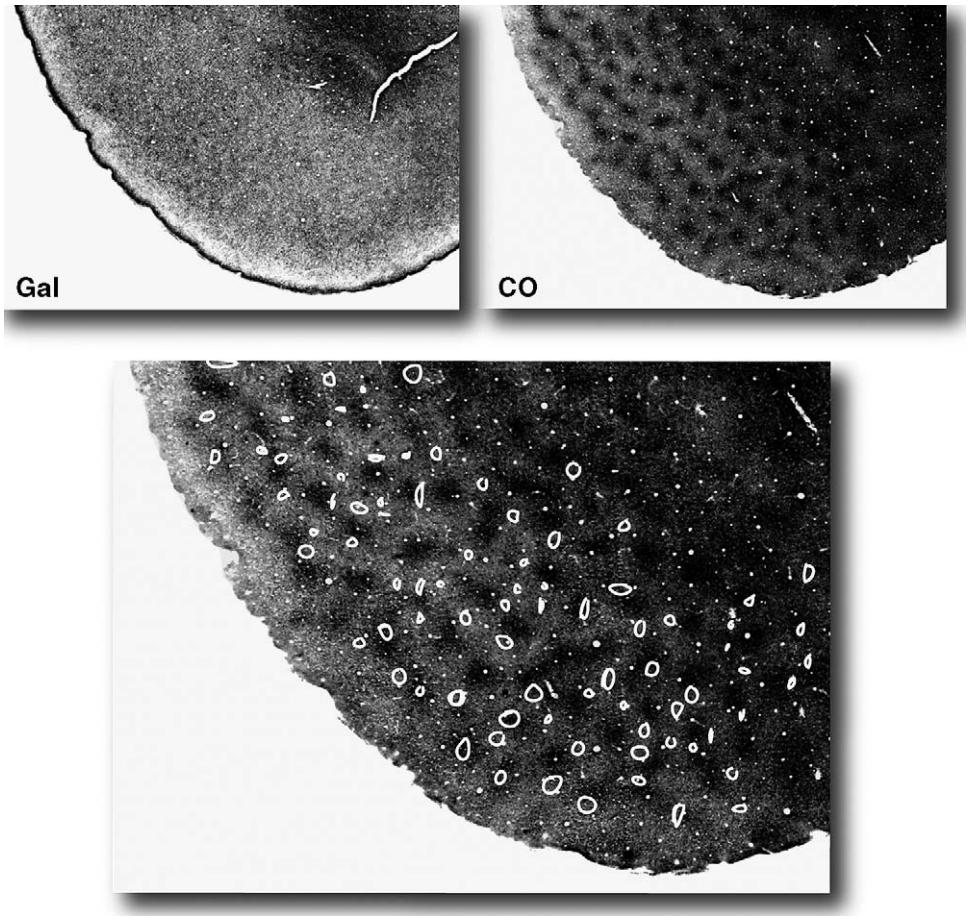

Fig. 7. Patterns revealed in flattened marmoset V1 using silver staining for myelin and cytochrome oxidase. Top, left is a section stained using the modified Gallyas technique. Top, right is an adjacent section stained for cytochrome oxidase, revealing a regular pattern of blobs. Bottom is an enlarged view of the CO section with a properly aligned overlay (white outlines) of the lightest areas in the Gallyas stain.

several blocks through the impregnation step at different times, as tissue became available. In another case, batches of sections from one block were impregnated at different times to control for sheer volume of tissue processed at once. Later, all tissue sections that were impregnated with silver nitrate were developed in the same container of solution at the same time, allowing multiple areas in the same brain or multiple brains to be stained with the same relative intensity.

The overall resolution of the staining found in the Gallyas silver stain (original and both the new and old modified forms) is much greater than that of the myelin basic protein antibody staining method, the Luxol Fast Blue method, Weil's rapid method, or Weigert's stain. The myelin basic protein method appears to have the greatest power of resolution only in areas sparsely stained for myelin using other techniques; but with the protocol we used the immunostaining saturates at a medium-to-high concentration of myelin and does not allow for distinction among middle and deep cortical layers and white matter. The Gallyas silver stain allows visualization of individual myelinated fibers throughout the brain, including cerebral cortex, where many other methods show only a contrast-difference in overall staining instead (more heavily-myelinated cortical areas stain darker). While the lightest areas in unstained wet sections viewed by darkfield illumination appear to correspond to the myelin-positive areas in multiple staining techniques, the resolution is not very high and it is impossible to be certain that one is exclusively illuminating myelin. The results indicate there may be no one technique suitable for viewing myelinated fibers in all regions of the CNS but, rather, that studies requiring visualization of myeloarchitecture should take advantage of multiple techniques tailored to specific areas of interest.

4.1. Limitations of the new modification

This modification simplifies the Gallyas silver stain for ease of use and increased quality and consistency of results, yet it still has several limitations. First, for those unfamiliar with pyridine, it requires the use of a fume hood and glassware, because it introduces a potential exposure hazard. Second, making and using the developer solution can still be difficult and it must be carefully monitored for color changes throughout the staining process, such as black or grey precipitates or a general cloudy or milky appearance to the solution. These precipitates tend to cause uneven and non-specific staining in sections, and their appearance should prompt immediate removal of the sections from the developer solution (if uneven staining resulted, the sec-

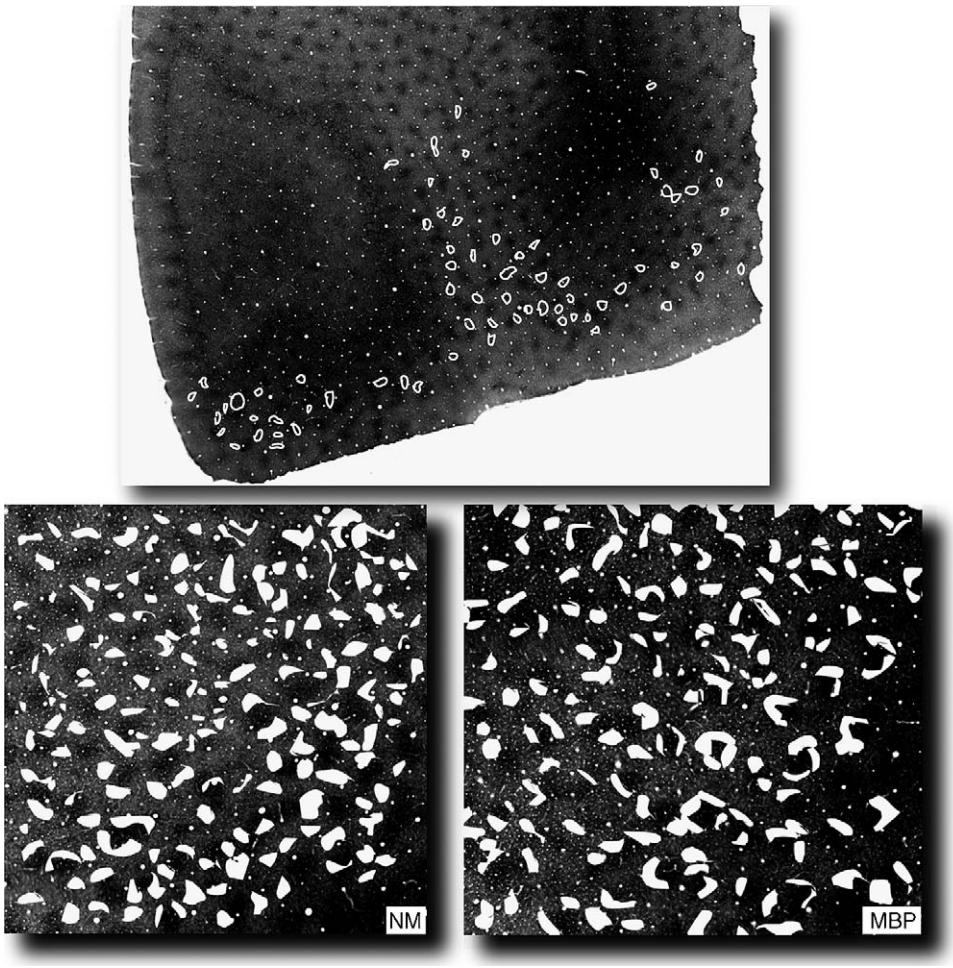

Fig. 8. Pattern revealed in macaque V1 using silver staining and immunohistochemistry for myelin, and cytochrome oxidase. Top is a section stained for CO with properly aligned overlay (white outlines) of the lighest areas in the new myelin stain. Bottom left is a close view of a CO section with an overlay (white shapes) of the darkest areas in the new myelin stain (NM). Bottom right is a close view of a CO section with an overlay (white shapes) of the darkest areas in the myelin basic protein stain (MBP).

tions can be completely bleached with ferricyanide, and then the developing process should be started again with freshly made developer). Third, final results require significant effort over a period of days. Despite these limitations, the ability to hold silver-impregnated tissue, processed at different times and/or from different samples, in 10% formalin for simultaneous physical development generally results in consistent relative intensity of staining between samples. Some factors that could impede consistent development across different tissue samples include post-mortem putrefaction, method and reagents used for fixation, and tissue storage time.

The relationship between myelin and CO staining of monkey V1 varies as much among studies as among species (Tootell et al., 1983; Krubitzer and Kaas, 1989, 1990; Horton and Hocking, 1997). Our results with both physical chemical and immunocytochemical methods demonstrate something more complex than coincidence of myelinated regions with blobs or inter-blobs. The pattern of myelin staining in flattened V1 of both marmoset and macaque was complex and on a much finer scale than the regular pattern seen in CO staining. The predominant pattern, which is the same for the species of New World monkey and the species of Old World monkey used in this study, was one in which the areas stained most darkly for myelin occupy the edges of blobs. Because neither the most darkly-stained areas nor the most lightly stained areas are always in or outside a blob, there seems to exist a more complex relationship between myelinated axons and blobs than previously suggested. For the immediate purposes of the present study, the comparison in V1 between the Gallyas stain, the new myelin silver stain, and myelin basic protein immunohistochemistry revealed similar patterns, suggesting that there are no anomalous results between properly performed techniques.

One must weigh the requirements of the study to determine the applicability of the method for myelin staining presented here. Any cytoarchitectonic study would benefit from the level of detail achieved by this method. Also, any anatomical or physiological investigation of sensory cortical areas would find this modification useful because of the level of detail found in cerebral cortex, as well as the ability to distinguish primary from non-primary sensory areas. Additionally, for investigators

researching neurological diseases, like multiple sclerosis, where demyelination is involved, this high-resolution stain may prove useful.

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