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An autometallographic technique for myelin staining in formaldehyde-fixed tissue

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Summary. A new autometallographic (AMG) technique for staining myelin in formaldehyde- or paraformaldehyde- (PFA) fixed tissue is presented. The tissue sections were exposed to AMG development without prior treatment with silver salts. The method was examined on PFA-fixed tissue from mouse, rat, pig, and formaldehyde-fixed human autopsy material. Samples from brain, spinal cord, cranial, and spinal nerves were either cut on a vibratome, frozen and cryostat sectioned, or embedded and microtome sectioned, before AMG development and counterstaining.

The AMG-myelin technique results in a specific black/dark-brown staining of myelin in all parts of the CNS and PNS. It works on all species examined, independent of the histological preparation techniques applied. The AMG staining is stable, stays unchanged through decades, allows counterstaining, and has previously been used with immunohistochemical techniques. On perfusion-fixed tissue the technique works without further fixation, but the intensity of the AMG-myelin staining is increased by increased postfixation time. Additionally, immersion fixation has to last for days depending on the size of the tissue block in order to obtain proper myelin staining.

The most feasible explanation of the chemical events underlying the AMG-myelin technique is that nano-sized clusters of metallic silver are formed in the myelin as a result of chemical bounds with reducing capacity, exposed or created by the formaldehyde molecule.

The AMG method is simple to perform and as specific as the conventional osmium and luxol fast blue stainings. The present technique is thus an effective, simple, inexpensive, and quick myelin staining method of formaldehyde- or PFA-fixed tissue.

Key words: AMG, Autometallographics silver enhancement, Fiber tracts, Formaldehyde, Paraformaldehyde

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Introduction

Catalytic clusters, consisting of a few atoms of gold or silver, can be silver-enhanced by autometallography (AMG) to sizes where they become visible in the light or electron microscope. This is caused by the capability of nano-sized gold or silver clusters (crystal lattices) to catalyze the reduction of silver ions on their surfaces, i.e. silver ions intimately connected to the crystal are reduced by hydroquinone molecules in the AMG developer. In this way a growing shell of metallic silver encapsulating the original catalytic cluster is formed (Danscher, 1981a,b; Danscher et al., 2002).

In 1983 we found that AMG development of semithin Epon sections of glutaraldehyde- and osmium-fixed tissue that had been placed in a 0.5% silver lactate solution for 30 min resulted in a contrast-rich staining of all membranes, including the double nuclear membrane, basement membranes, the interior of certain lysosomes, and secretory vesicles (Danscher, 1983). This "AMG-silver-lactate" counterstaining technique revealed details in the light microscope normally visible only at low EM magnifications. Additionally, myelin was heavily stained. However, recently we have observed that myelin stained black/dark-brown after AMG development if the tissue had been fixed in formaldehyde or PFA without additional pretreatment. Perfusion or immersion fixation with formaldehyde or PFA was thus able to build up a chemical capacity in the myelin to catalyze AMG silver enhancement. The AMG-myelin technique offers a remarkably clear differentiation between gray and white matter in the brain, enabling a clear segregation of the gross anatomical structures in the CNS.

The AMG-myelin staining was examined with regard to species specificity, histological processing, and the optimal formaldehyde fixation criteria.

Materials and methods

AMG staining protocol (for details, see table 1 and related text): 1) The tissue was fixed either by transcardial perfusion or by immersion in 0.15 M phosphate-buffered PFA or formaldehyde. 2) The fixed

tissue blocks were either frozen and cut on a cryostat, or embedded in paraffin, methacrylate or Epon and cut in histological sections. 3) All sections were placed on Farmer-rinsed glass slides and placed in jars and covered with the AMG developer. 4) The sections were developed for 1-2 hrs in a water bath at 26 ºC covered by a light-tight lid. 5) The development was stopped by replacing the AMG developer with 5% thiosulphate for 10 min. The glass slides were then rinsed several times in distilled water. 6) Counterstaining was performed with 0.1% toluidine blue in citrate buffer, pH 4.0. 7) Finally the sections were rinsed twice in distilled water, dehydrated in 99% alcohol, imbibed with xylene, mounted with Depex, and coverslipped.

The AMG developer was prepared according to Danscher (1981b) from the constituents listed below by carefully mixing 60 ml of solution I with 10 ml of solution II and 15 ml of solution III. Just prior to the development the silver ion solution (IV) was added.

I. Protective colloid: One kg of crystalline gum arabic was dissolved in 2 l deionized water for 5 days with intermittent stirring 8-10 times a day. The solution was then filtered through 1, 2, and 3 layers of gauze, respectively. If not used immediately, the solution can be frozen in plastic jars and stored for more than a year.

II. Citrate buffer: 25.5 g citric acid monohydrate + 23.5 g sodium citrate. Distilled water was added to make 100 ml.

III. Reducing agent: 0.85 g hydroquinone dissolved at 40 ˚C in 15 ml heated distilled water.

IV. Silver ion supply: 0.10 g silver lactate was dissolved in 15 ml distilled water at 40 ˚C. The solution should be protected from light, e.g. by wrapping the vial in aluminum foil.

All animals were anesthetized with a combination of midazolam and ketamine or pentobarbital before sacrifice as approved by the Danish Council for Animal Research Ethics. PFA and formaldehyde were buffered to pH 7.4 with a 0.15 M phosphate buffer. Specimens for cryostat cutting were immersed in 30% sucrose for 24 hrs, prior to being frozen with CO_2 gas or dry ice.

The brain samples were cut frontally and the spinal cord, sciatic, trigeminal, and optic nerves were cut transversally. All sections were fixed to glass slides before AMG development. Tissue for EM was cut on the vibratome in 100 μm-thick sections and AMG developed before Epon embedding and ultrathin sectioning (for details see Danscher et al., 2002). If ultrastructural studies are important to the experiment, we recommend that the animal is perfused with a buffered combination of glutaraldehyde and PFA for 15 min and allowed to postfix in this fixative for two days before further processing.

The developing time needed for an optimal myelin staining depends on the fixation method and the histological processing. In our hands, cryostat sections from transcardially perfused animals stained beautifully if developed for 60 min, whereas immersion-fixed animals needed 75 min, methacrylate-embedded sections 70 min, and paraffin- and Epon-embedded sections 120 min for a satisfactory result.

Unfixed and glutaraldehyde-fixed sections served as controls. Selected cryostat and paraffin sections were incubated with a 1% osmium solution for 1-24 hrs for comparison. Additionally, paraffin sections were stained with a conventional luxol fast blue method (Romeis, 1989).

Results

AMG development of formaldehyde-fixed tissue resulted in a specific black/dark-brown myelin staining in all analyzed parts of the nervous system (Fig. 1A-C) independent of the examined species (Fig. 1A-F). The staining could be observed at the macroscopic level as well as at LM and EM levels (Figs. 1, 2). No staining of neurons, axons, or glia was observed, neither in the CNS nor the PNS. The resulting myelin staining was not influenced by the consecutive counterstaining with toluidine. The AMG staining method stained myelin as

Table 1. Species used and tissue processing.

SPECIES	FIXATION	SAMPLES	POSTFIXATION PERIOD	PROCESSING	AMG-DEVELOPING TIME
5 Göttingen minipigs	4% PFA transcardial	brain, spinal cord	36 weeks	cryostat, 40 μm	60 min
1 Göttingen minipig	1% glutaraldehyde+ 1% PFA transcardial	brain, spinal cord, optic & sciatic nerve	3 weeks, 6 weeks, 12 weeks	cryostat 40 μm, paraffin 5 μm	60 min 120 min
1 Balb/c mouse	4% PFA transcardial	brain	4-5 days	methacrylate 30 μm	70 min
2 NMRI mice	4% PFA transcardial	brain, spinal cord, sciatic & trigeminal nerve	4-5 days, 6 weeks	Epon, electron microscopy 50-100 nm	120 min
1 Wistar rat	4% PFA transcardial	brain, spinal cord	5 days	cryostat, 40 μm	60 min
1 Wistar rat	4% PFA immersion 0, 8, 24, 48 and 72 hrs after sacrifice	brain, spinal cord	4-5 days	cryostat, 40 μm	75 min
1 human brain	10% formaldehyde immersion	hippocampus, pons, prefrontal cortex	14 months	vibratome, 50 μm paraffin 5 μm	75 min 120 min

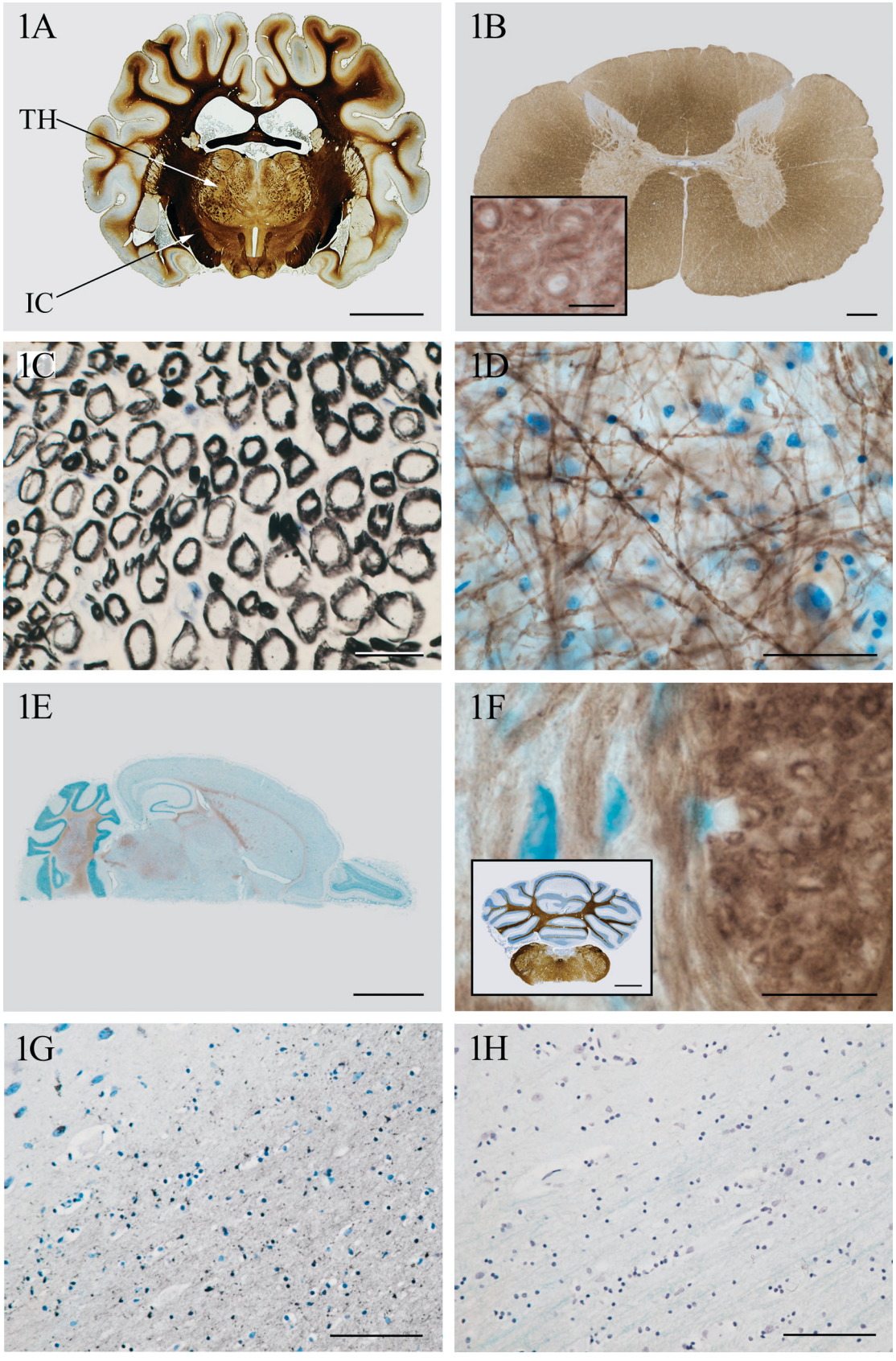

Fig. 1. A. Frontal 40 μm-thick cryostat section of the pig brain transcardially perfused with 4% PFA and postfixed for 36 weeks. TH: thalamus, IC: internal capsule. Scale bar: 10 mm. **B.** Horizontal 40 μm-thick cryostat section of the pig spinal cord transcardially perfused with 1% PFA and 1% glutaraldehyde, and postfixed for 12 weeks. Heavily stained myelin sheaths encircle the unstained axons (inset). Scale bar: 1 mm, inset 30 μm. **C.** Transverse 5 μm-thick section of paraffin-embedded pig sciatic nerve, after perfusion with 1% PFA and 1% glutaraldehyde. Scale bar: 30 μm. **D.** 50 μm-thick vibratome section of the hippocampal region of a human autopsy brain, immersion fixed in 10% formaldehyde for 14 months. Scale bar: 5 μm. **E.** Horizontal section of a mouse brain, halved in the sagittal plane. The animal was perfused transcardially with 4% PFA, methacrylate-embedded after 4 days of postfixation, and cut into 30 μm-thick sections. Scale bar: 2 mm. **F.** Frontal 40 μm-thick cryostat section of the rat brainstem and cerebellum perfused transcardially with 4% PFA and postfixed for 5 days. Longitudinal and transversally cut fibers can be observed. Scale bar: 20 μm, inset 2 mm. **G:** Frontal 5 μm-thick paraffin section of the prefrontal cortex of a human autopsy brain, immersion-fixed in 10% formaldehyde for 14 months. The section depicts the border between gray (upper left corner) and white substance. Scale bar: 100 μm. **H:** Material as in figure G but stained with the luxol fast blue method. Scale bar: 100 μm.

specifically as the conventional methods as displayed with the luxol fast blue (Fig. 1G-H). No myelin staining was seen in unfixed and glutaraldehyde-fixed control sections.

The quality of the myelin staining was independent of the different histological procedures used (Figs. 1-2). The necessary development time was, however, influenced by the fixation method and the histological processing, since un-embedded sections from transcardially fixed animals needed shorter developing times than immersion-fixed and embedded sections. It should be noted that increased developing times may

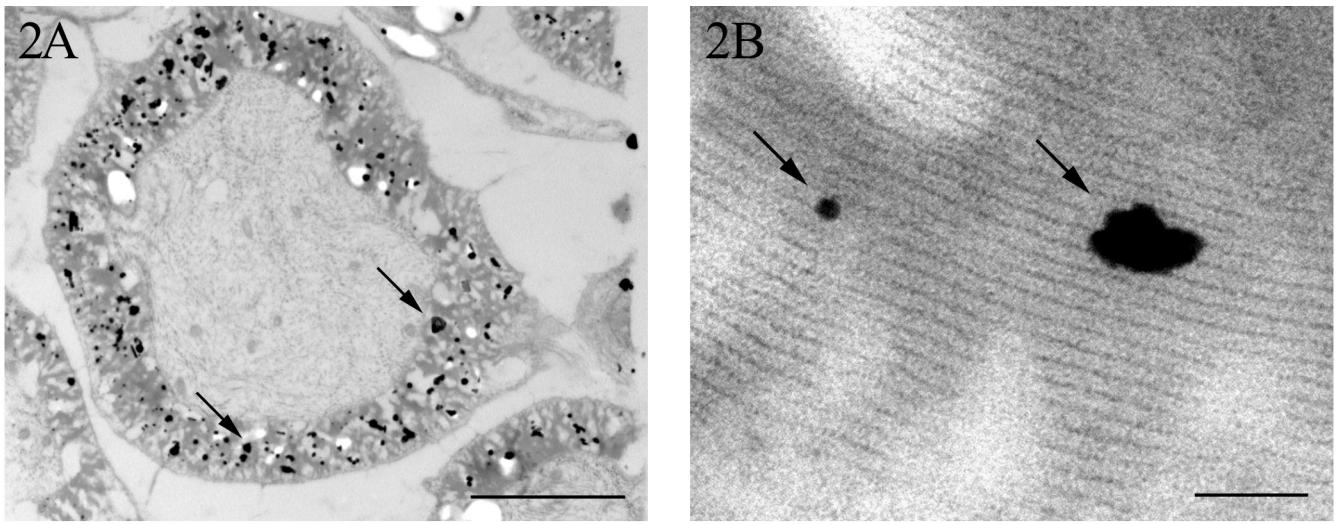

Fig. 2. A. Electron microscopic picture of a transected mouse trigeminal nerve cut to 50-100 nm thick. The animal was transcardially perfused with 4% PFA and postfixed for 6 weeks. AMG silver grains (arrows) are located specifically in the myelin sheath. Scale bar: 2 μm. **B.** The myelin sheath, revealing the lamellar structure of the myelin and the AMG silver grains (arrows). Scale bar: 0.1 μm.

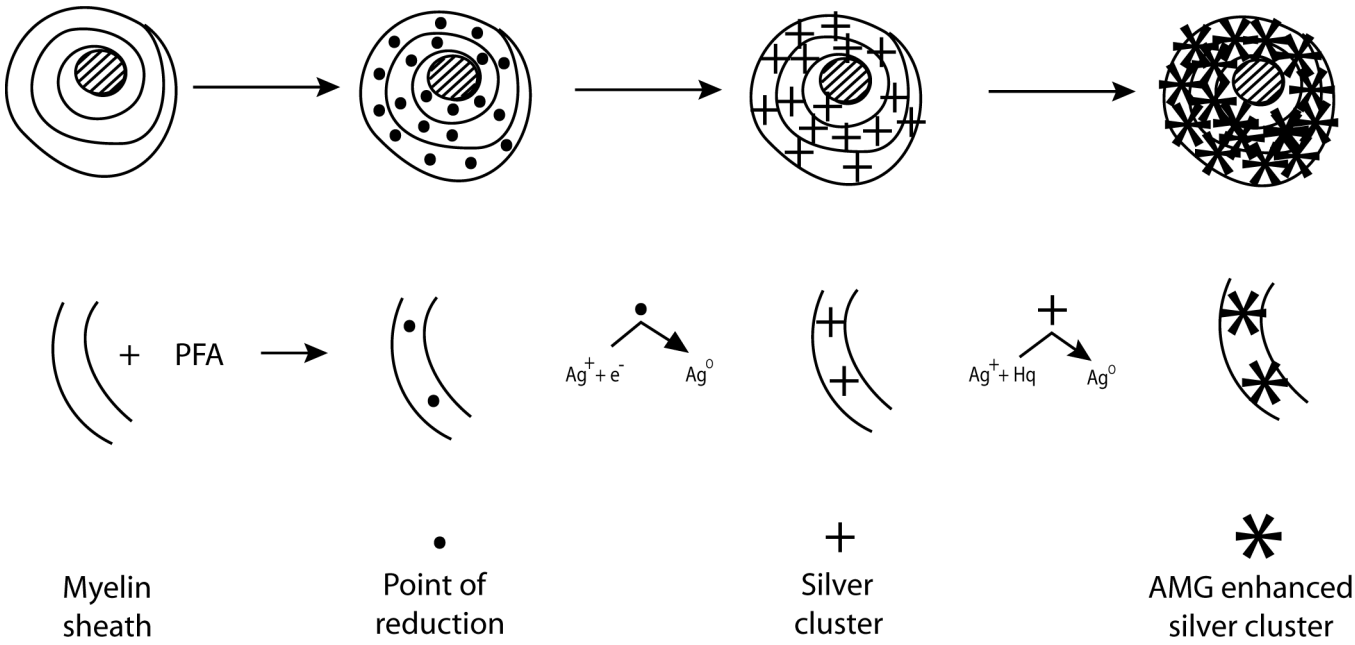

Fig. 3. Schematic drawing depicting the supposed mechanism of the new AMG-myelin stain. PFA or formaldehyde reacts with the myelin sheath, creating "points of reduction" (•). During the initial AMG development phase silver ions in the AMG developer are bound to these points in the myelin and reduced to metallic silver, creating catalytic silver clusters (+). These are able to bind more silver ions in the later stages of the AMG development, and these silver ions are reduced to visible AMG enhanced silver clusters (*) in the presence of hydroquinone (Hq).

increase the risk of unspecific background staining. If it is necessary to use long developing times, i.e. more than 80 min, we recommend dipping the glass slides in a 0.5% gelatin solution and subsequent drying for 15 min before AMG development. After development the gelatin coat can be removed by a gentle rinse in 40 ºC tap water for 15 min. Another way to reduce unwanted background staining is to dip the glass slides in a Farmer solution for a few seconds after AMG development (Danscher et al., 2002). While perfusion-fixed tissue sections showed AMG-myelin staining without further fixation, immersion-fixed tissue needed postfixation for 4-5 days for a satisfactory staining result. Electron microscopic studies of Epon-embedded mouse trigeminal nerves confirmed that the AMG silver grains were located in the membranes that form the myelin sheath (Fig. 2).

Discussion

This study describes a specific myelin staining of formalin- or PFA-fixed nervous tissue based on the AMG technique. In the present study the AMG-myelin staining technique was tested in pig, mouse, and rat specimens as well as human postmortem material, myelin being demonstrated in all assessed histological preparation methods.

Tissue fixation with formaldehyde or PFA seems to cause a chemical build-up of "points of reduction" in the myelin membrane (Fig. 3). If these points are flooded with silver ions contained in the AMG developer, they will cause a build-up of clusters of metallic silver atoms on the spot. The tiny clusters of silver initially formed will catalyze the binding of new silver ions to these clusters, and the silver ions will be reduced to metallic silver in the presence of hydroquinone (likewise contained in the AMG developer), resulting in a visible AMG-silver grain (Fig. 3). Another explanation of the AMG-myelin technique could be that formaldehyde fixation exposes tightly spaced hydrogen sulphide bonds in the myelin that then bind silver ions. Such clusters will also catalyze the AMG process of silver enhancement in the presence of silver ions and hydroquinone from the AMG developer.

Silver staining of myelin by physical development has been described by Gallyas (1979). Gallyas suggested that myelin could form and bind colloidal silver particles if placed in a 0.1% ammoniac silver nitrate solution at pH 7.5. However, this staining method also requires a suppression of the production of metallic silver by other tissue elements by treating the sections with a 2:1 mixture of pyridine and acetic anhydride. The AMG-myelin staining method differs from this method in two ways: 1) the reducing capacity of myelin is enhanced by fixation in PFA or formaldehyde; and 2) the AMG developer delivers silver ions both for the myelin reduction and the concomitant silver enhancement of the catalytic silver clusters created by the fixed myelin.

Compared with conventional myelin staining methods, the AMG method offers several advantages. This method stains myelin as specifically as the conventional osmium and luxol fast blue, and the myelin stain may be performed within 1-2 hrs, thus avoiding overnight staining required for the luxol fast blue. The AMG method does not require toxic chemicals like the conventional methods, and is inexpensive compared with the osmium method.

In conclusion, we propose the new AMG-myelin staining technique as an interesting alternative to conventional myelin stains such as osmium, luxol fast blue, iron-hematoxylin, and thionin (Weigert, 1884; Klüver and Barrera, 1953; Benes et al., 1985; Kutscher, 1987; Romeis, 1989).

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References

Benes E.S., Vaupel M.R. and Bailey S.B. (1985). A simplified technique for preparing sections of osmium stained myelin sheaths. Stain Technol. 60, 114-116.

Danscher G. (1981a). Localization of gold in biological tissue. A photochemical method for light and electronmicroscopy. Histochemistry 71, 81-88.

Danscher G. (1981b). Light and electron microscopic localization of silver in biological tissue. Histochemistry 71, 177-186.

Danscher G. (1983). A silver method for counterstaining plastic embedded tissue. Stain Technol. 58, 365-372.

Danscher G., Hacker G.W. and Stoltenberg M. (2002). Autometallographic tracing of gold, silver, bismuth, mercury and zinc. In: Gold and silver staining. Techniques in molecular morphology. Hacker G.W. and Gu J. (eds). CRC Press, Boca Raton, pp. 13-27.

Gallyas F. (1979). Silver staining of myelin by means of physical development. Neurol. Res. 1, 203-209.

Klüver H. and Barrera E. (1953). Method for the combined staining of cells and fibers in the nervous system. J. Neuropathol. Exp. Neurol. 12, 400-403.

Kutscher C.L., Cheek M., Borski K. and Maucher J. (1987). Hematoxylin and thionin techniques for staining myelin and cells: variations and critical steps. Brain Res. Bull. 19, 161-163.

Romeis B. (1989). Mikroskopische Technik. München, Wien, Baltimore:

Urban & Schwarzenberg.

Weigert C. (1884). Ausführliche Beschreibung der in no. 2 dieser Zeitschrift erwähnten neuen Färbungsmethode für das Centralnervensystem. Fortschr. Deutsch. Med. 2, 190-192.

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