



# Retro is the new modern: Contemporary application of gold impregnation staining on brain cryosections for digital image analysis

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## Abbreviations:

CA1-3 – cornu Ammonis 1-3

cg – cingulum

CPu – caudate putamen

DG – dentate gyrus

Hb – habenula

IHC – immunohistochemistry

M1 – primary motor cortex

Pir – piriform cortex

ROI – region of interest

S1BF – primary somatosensory cortex, barrel field

SuG – superficial grey layer of the superior colliculus

V1M – primary visual cortex – monocular area

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## Abstract

**Background and purpose:** Since the times of Golgi and Cajal, impregnation with gold or silver has been used to visualize microscopic details of the nervous tissue. Although immunohistochemistry has largely replaced impregnation techniques, they are still used, and there is a growing interest in combining them with modern image analysis methods for quantitative studies in neuroscience. The aim of this research was to modify the gold chloride impregnation method published by Schmued to improve consistency of staining, to be adequate for digital image analysis.

**Materials and methods:** Brains of 8 six-month-old female Wistar rats were fixed in 4% PFA, cryoprotected in sucrose and flash-frozen in liquid nitrogen. Neighboring sets of coronal sections were chosen for gold impregnation, Nissl staining and MAP2 immunohistochemistry. Whole-slide images and images of specific regions were taken for analysis.

**Results:** Myelinated fibers were stained dark reddish to brown on gold-stained sections, and other tissue was yellowish, which gave an excellent contrast for digital image analysis. Gold staining was consistent in all regions, and no major artifacts were noticed. When compared to Nissl and MAP2, only myelinated structures were stained with gold impregnation.

**Conclusions:** Modified gold impregnation method is an alternative that's on par with traditional myelin staining methods. The new, modified gold impregnation method gives a consistent and reproducible staining suitable for digital image analysis. It can be useful in morphometric evaluation of nervous tissue and investigation of neuropathological changes in nervous tissue, especially for quantitative studies.

## INTRODUCTION

### Nervous tissue histology

Nervous tissue consists of neurons and glial cells. Neurons have the perikaryon (or cell body, that contains the nucleus and cytoplasm with most of the cell's organelles), dendrites and the axon. The cytoplasm of the neuronal cell body and processes contains microtubules, actin filaments and neurofilaments. Axons can be myelinated or unmyelinated, depending on whether they are covered by a myelin sheath or not. Histological studies of nervous tissue are still an essential tool in modern neuroscience, often coupled with modern microscopes and digital cameras. Depending on the desired result, nervous tissue can be

stained using different techniques. Examples include the frequent use of Nissl (cresyl violet) staining for visualization of the cell bodies, as well as other staining techniques for cellular processes and immunohistochemical (IHC) methods for identifying specific protein targets in neurons and glial cells. Special staining protocols also exist for detecting specific neuropathological changes in certain diseases, such as Alzheimer's disease. Some of the oldest types of staining that were used in microscopic examination of the nervous tissue are impregnation techniques, which are still being used today (1).

### **Impregnation staining techniques in neuroscience**

Silver impregnation, perfected by Camillo Golgi, enabled the understanding of complex neuroanatomical microarchitecture (2). Golgi and Santiago Ramón y Cajal, who mastered the use of Golgi's method, came to opposite conclusions about nervous tissue building elements (reticular and cellular theory) (3). Despite this, both scientists received a well-deserved Nobel Prize in 1906, because silver staining proved to be the most powerful tool in neuroanatomy at the beginning of the twentieth century, leading to many discoveries in neuroscience (4). The method itself was far from perfect, because the final precipitating reaction – reduction of metallic silver – was hard to control, so it was hard to predict which cells would be stained and obtaining consistent results was a challenge. To make the method more predictable, its numerous variations were invented, e.g., optimization for neurofibrillary staining for neuropathology use, published in 1908 by Bielschowsky; later Bodian optimized the method for paraffin slides, and the most important variations of the method were made by Gallyash, who also exposed the basic principles of nerve tissue staining and perfected the silver method for staining of different brain structures (5).

Among the first gold impregnation techniques of nervous tissue were Conheim's method for peripheral nerve fibers and a modification of Golgi's method by Ramón y Cajal using gold chloride-sublimite (6,7). Gold has been used for toning neurons previously impregnated using silver (7). Gold chloride staining, especially with its improvements over the last century, has become known as an important myelin staining method, because it stains myelinated axons and sensory nerve endings well (8). Nevertheless, over the years it has become apparent that using gold chloride is prone to inconsistent staining results, which is why recent modifications of gold impregnation staining were developed with better consistency in mind (8,9). Gold chloride has been used in nervous tissue studies both for impregnation of frozen tissue sections and whole tissue parts (*en bloc*) (10).

For some time, IHC has largely replaced impregnation techniques, but many still use them when quantitative studies are needed (1). With the development of modern

imaging and image analysis methods, these "old" impregnation methods are once again revisited in the context of modern neuroscience and nervous tissue visualization and morphometry, while also being supplemented with various modern methods (11–14). In our paper, we demonstrate a new and modified gold chloride impregnation method on rat brain cryosections to visualize nerve fibers in different regions. We significantly modified the method published by Schmued (7) to increase the reproducibility and consistency of staining. We also stained the corresponding sections using Nissl method and IHC for Microtubule-associated protein 2 (MAP2) for morphological comparison.

## **MATERIAL AND METHODS**

### **Use of animals**

This research was approved by ethical committees of Faculty of Science, University of Zagreb (Approval Code: 251-58-10617-19-704) and Faculty of Medicine Osijek (Approval Code: 2158-61-46-22-16), as well as Croatian National ethical committee for protection of laboratory animals (EP 233/2020). Care and handling of experimental animals followed mandatory ethical guidelines and legislature (Croatian Law on the Welfare of Animals, NN135/06 and NN37/13, EU Directive 2010/63/EU for animal experiments and National Research Council (US) Committee's Guide for the Care and Use of Laboratory Animals).

### **Sample preparation**

We used eight six-month-old female Wistar rats for the gold impregnation method testing. All animals were anesthetized, sacrificed and brains were harvested after craniotomy.

Solutions for tissue fixation were buffered with phosphate buffered saline (PBS) at pH=7.40. Rat brains were submerged in 4% paraformaldehyde (PFA) (Acros Organics, Geel, Belgium) solution for 72 hours at 4°C. After fixation, brains were cryopreserved in ascending sucrose (Thermo Fisher, Waltham, United States) solution gradient (10%, 20%, 30%). Following cryopreservation brains were snap frozen in liquid nitrogen (5-6 seconds) and stored at -80°C until further processing. Brains were cut on a Leica CM3050S cryostat (Leica, Wetzlar, Germany) into 35 µm thick coronal sections, which were kept in cryoprotectant solution (30% sucrose, 40% glycerol and 0.9% formaldehyde in 1× phosphate buffer) and stored at -20°C.

### **Gold impregnation staining procedure**

We selected three different sections from each animal for gold impregnation staining based on their interaural (IA) and Bregma (BG) stereotaxic coordinates (IA 8.74 / BG -0.26; IA 6.20 / BG -2.80; IA 3.20 / BG -5.80). Prior

to staining, sections were placed in 12 well plates filled with PBS and washed 3 times with cold PBS before mounting on glass slides. The slides were coated with gelatin for better adhesion (Gelatin Type A, Sigma-Aldrich, St. Louis, USA). Gelatin solution was prepared at 5 g/L concentration with the addition of 0.5 g/L chromium potassium sulfate dodecahydrate (Sigma-Aldrich, St. Louis, USA). Slides were placed in a slide rack and submerged 5 times in the gelatin solution, around 3 seconds each time. After submersion, excess gelatin solution was drained off and slides were left to dry for 48 hours before use. Brain sections were mounted on glass slides in distilled water. Each slide was submerged in distilled water and sections were mounted one by one using a thin paintbrush. After mounting, sections were left to dry overnight in a dust-free area. Next day the mounted sections were rinsed in distilled water two times for 5 minutes to flush any remaining salts from the sections.

We prepared all solutions used in staining procedure in double-distilled water. Stock solutions were prepared in advance: 0.5% (m/v) chloroauric acid ( $\text{H}(\text{AuCl}_4)_x \cdot n\text{H}_2\text{O}$ ); (Kemika, Zagreb, Croatia), sodium phosphate buffer prepared by mixing 39 parts of 1M  $\text{NaH}_2\text{PO}_4$  (VWR, Radnor, United States) and 61 parts of 1M  $\text{Na}_2\text{HPO}_4$  (VWR, Radnor, United States) resulting in pH=7.00 buffer stock, and 5M NaCl (T.T.T., Sveta Nedelja, Croatia). Chloroauric acid solution must be stored in an amber glass bottle at 4°C and it is stable for at least 12 months in these conditions. A humid chamber for slides was prepared and rinsed slides were placed inside the chamber. A working solution for staining was prepared with final concentrations of 0.2% chloroauric acid, 20 mM phosphate buffer, and 150 mM NaCl. We performed the preparation of working solution in dimmed-light conditions to prevent photolytic reaction. The solution was applied on glass slides to completely cover the entire slide. The slides were placed in a dark room (or, optionally, in a drawer) for 2.5-3 hours. Fluorescent lights were turned off and LED lights used instead to check the progress of staining. After the staining, the slides were rinsed 3 times with deionized water, dehydrated, cleared in xylene, and coverslipped using resinous DPX mounting medium Biomount (Biognost, Zagreb, Croatia).

### **Nissl staining procedure**

We selected three different sections with similar stereotaxic coordinates as gold impregnated sections from each animal. Sections were washed 3 times with cold PBS and mounted on gelatinized slides. After drying they were washed in distilled water to remove residual salts. Acidified 0.1% cresyl-violet stain (Sigma-Aldrich, St. Louis, USA) in 20% ethanol was prepared and used to stain slides for 10 minutes. Slides were quickly washed 2 times in 96% ethanol followed by 2 quick washes in 100% ethanol, cleared in xylene and coverslipped using Biomount medium (Biognost, Zagreb, Croatia).

### **MAP2 IHC staining procedure**

For MAP2 IHC, we also selected three different sections with similar stereotaxic coordinates as gold impregnated sections, and we used one extra section from each animal and washed in cold PBS in 12 well plates. After washing, sections were incubated 2 times for 20 minutes in 1% hydrogen peroxide (Gram-Mol, Zagreb, Croatia) solution to inhibit endogenous peroxidase and further washed with PBS. Sections were incubated overnight at +4°C in blocking solution (goat/bovine serum in PBS) with MAP2 primary antibody diluted 1:1000 (M4403, Sigma-Aldrich St. Louis, USA) and extra section was incubated in blocking solution without primary antibody (negative control). After primary antibody incubation, sections were washed with PBS and placed in biotin labeled secondary antibody (111-065-071, Jackson-ImmunoResearch, West Grove, USA) solution diluted 1:500 in blocking solution and incubated for 2 hours at room temperature. Secondary antibody solution was washed out using PBS and sections were placed in streptavidin-HRP complex (Vector PK-600, Vector Laboratories, Burlingame, USA) solution diluted 1:250 in PBS. After 1 hour incubation and washing in PBS, the reaction was developed using DAB substrate kit (Vector DAB Substrate Kit, Vector Laboratories, Burlingame, USA) for 2 minutes. Excess DAB substrate was washed off in PBS, sections were mounted on gelatinized slides, dried and coverslipped using Biomount medium (Biognost, Zagreb, Croatia).

### **Image analysis**

Large, whole-slide images were taken using a motorized Axiovert 200M microscope (Carl Zeiss, Jena, Germany) and AxioCam MRC camera (Carl Zeiss, Jena, Germany) by taking series of images using a 10× objective and stitching them into whole-slide images of coronal sections. We also photographed regions of interest on gold impregnated sections using 20× objective. The following regions were examined: primary motor cortex (M1), primary somatosensory cortex – barrel field (S1BF), primary visual cortex – monocular area (V1M), piriform cortex (Pir), fields of hippocampus 1-3 (CA1-3), dentate gyrus (DG), cingulum (cg), caudate putamen (CPu), habenula (Hb) and superficial gray layer of the superior colliculus (SuG).

Isolation of gold-impregnated structures was performed on whole-section images and magnified images (20× objective) using free and open-source program FIJI, a distribution of ImageJ image analysis software (15, 16). Black-and-white mask depicting all gold impregnated structures in a brain digital image was created using Color Thresholding tool and selection was created (*Edit>Selection>Create Selection*). Selection was added to ROI manager (*Edit>Selection>Add to Manager*). We used the obtained selection to isolate gold-impregnated

structures from the original image after converting the image to 8-bit type (*Image>Type>8-bit*). Image prepared in this manner can be used to measure integrated density and/or mean gray value of the staining.

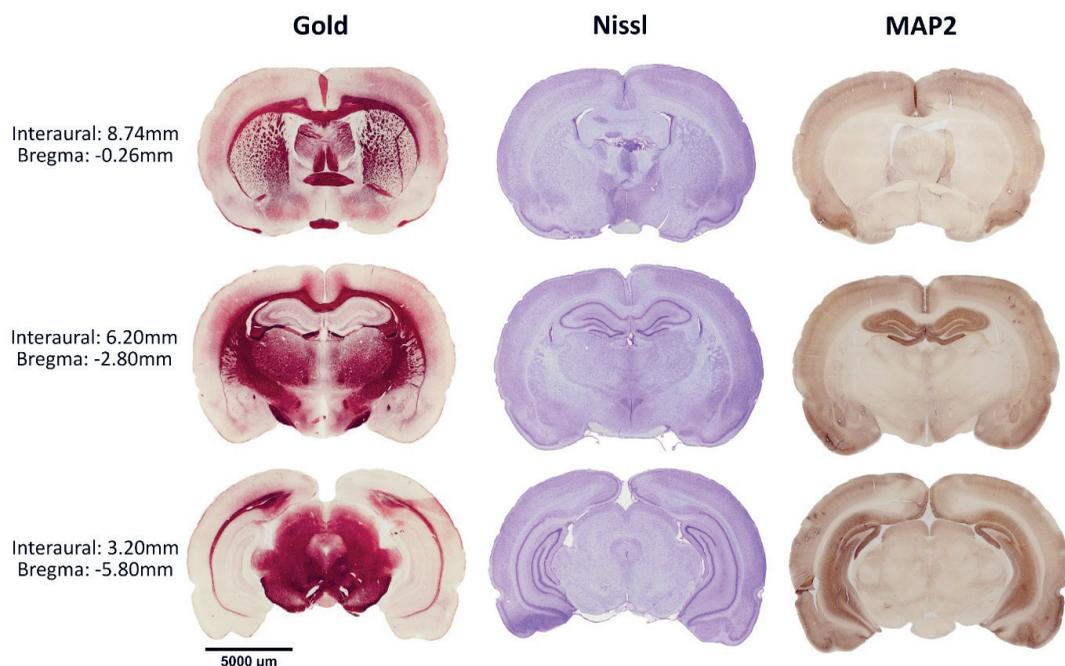
## RESULTS AND DISCUSSION

By brightfield microscope examination we found an excellent quality of nerve fiber impregnation in all examined regions of the brain stained using our new gold method. Myelinated fibers were stained dark reddish to brown, and the background and other tissue was yellowish in color, which gave an excellent contrast for further digital image analysis. We presented examples of coronal brain sections and comparison with Nissl staining and MAP2 IHC in Figure 1. Nissl method-stained cell bodies, and MAP2 IHC stained primarily the dendrites and only mildly the cell bodies, which was expected as it is normally present in the dendrites, areas of neuronal differentiation, and places of microtubule assembly. Because of this, Nissl and gold impregnation complement each other by emphasizing the different parts of brain regions and structures (like a “positive” and “negative” photograph).

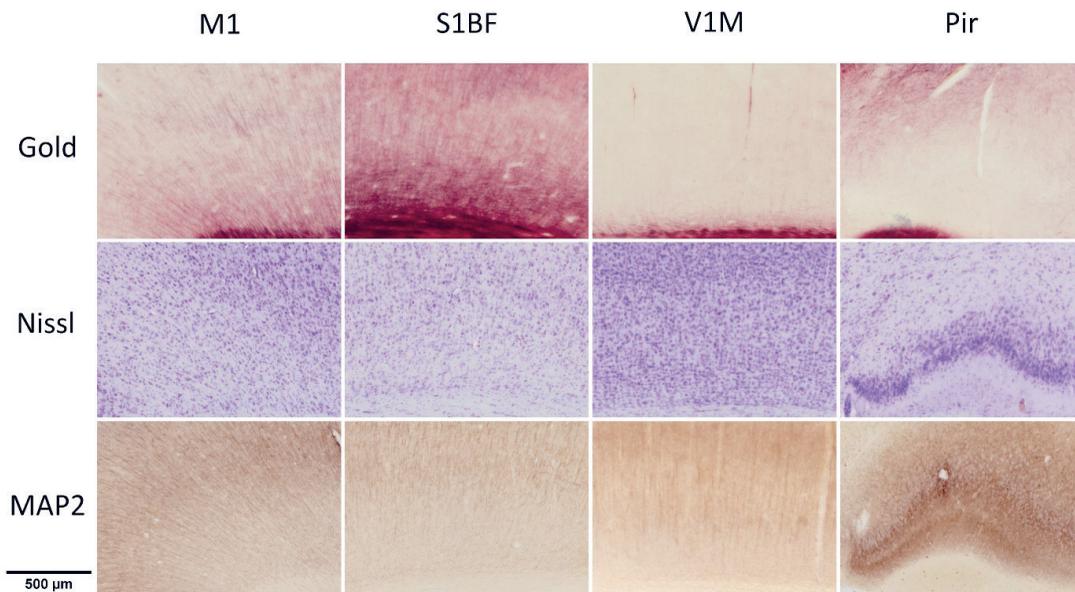
Gold staining was consistent in all regions, we found no significant artifacts, and the examined regions were consistent with the rat brain atlas (17). Staining of specific regions in the cortex, hippocampus, and the selected regions with high presence of myelinated axons was consistent with the other two methods. We have shown representative images of these regions in Figures 2–4. For

example, accumulations of cell bodies produced strong staining on Nissl-stained sections in the granular cell layer in DG and pyramidal layer of CA1-3, but they did not stain on the gold-impregnated sections (Figure 3). Molecular layer in different regions stained strongly for MAP2 (Figure 2 and 3). Also, in the CPu, large bundles of myelinated fibers were strongly stained with gold impregnation method, while the surrounding tissue dominated by cell bodies was not stained; on the other hand, the Nissl method stained the areas of CPu occupied by cell bodies, while the white matter showed only small nuclei of glial cells. On MAP2 IHC of CPu, a medium-intensity staining of the dendrites between the cell bodies is present, while the staining is absent in the areas of myelinated fiber bundles (Figure 4). Similar staining pattern is visible in other depicted brain regions, depending on their histological structure.

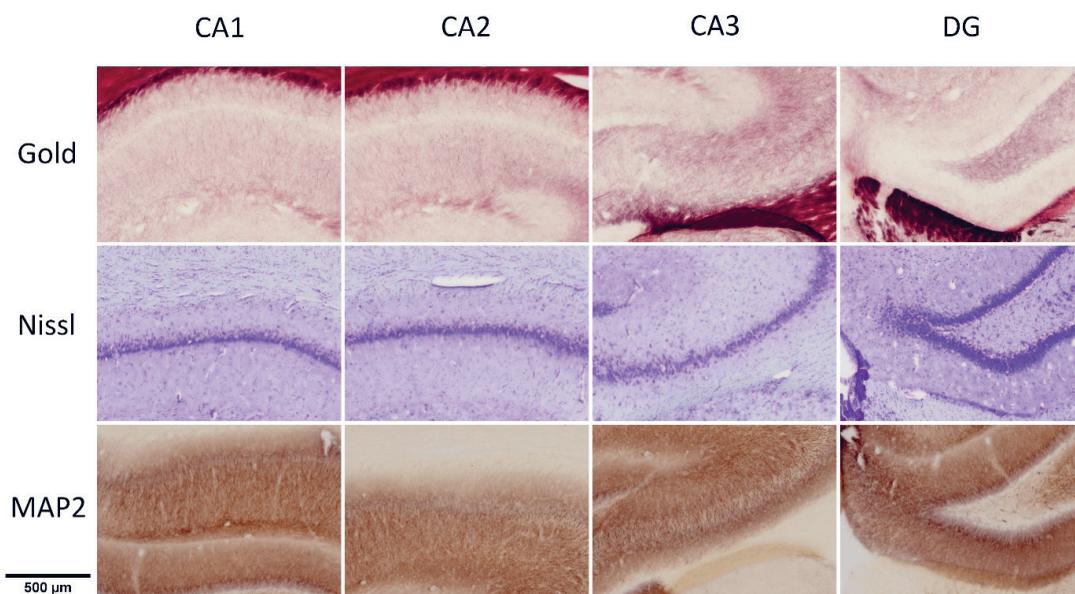
All the gold-impregnated tissue images were suitable for analysis in FIJI, color thresholding was successful, and after converting the images as described in the Methods section, integrated density could be calculated – a sum of the pixel values (between 0 and 255 for each pixel) in the image or selection. This can be used for comparison between the experimental groups in different morphological studies. Maximum value was calculated by multiplying the total pixel number in an image by 255 (maximum pixel value). For example, the whole-slide image in Figure 5 has 163,704,177 pixels, and the picture taken under 20× objective in Figure 5 has 1,443,520 pixels. Hence, the maximum integrated density for pictures of that size is 41,744,565,135 and 368,097,600, respectively, while in



**Figure 1.** Comparison among gold impregnation, Nissl stain and MAP2 IHC. The image shows coronal brain sections stained with the 3 methods, with approximate stereotaxic coordinates. 10× objective, scale bar: 5000 µm.



**Figure 2.** Regions of interest in the cortex: primary motor cortex (M1), primary somatosensory cortex – barrel field (S1BF), primary visual cortex – monocular area (V1M) and piriform cortex (Pir). Each region is stained using gold impregnation, Nissl stain and MAP2 IHC. 10× objective, scale bar: 500 µm.



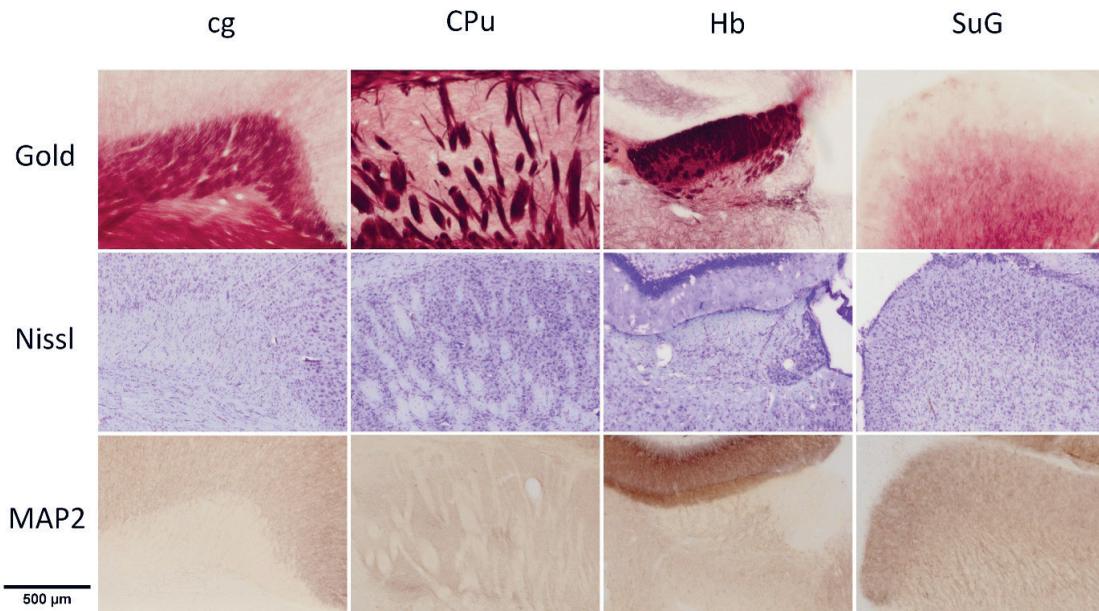
**Figure 3.** Regions of hippocampus stained using gold impregnation, Nissl stain and MAP2 IHC. Represented regions of hippocampus: cornu Ammonis 1-3 (CA1-3) and dentate gyrus (DG). 10× objective, scale bar: 500 µm.

our example the calculated density was 3,120,373,011 and 94,148,812, respectively (Figure 5). These numbers represent the intensity of gold impregnation staining on the slide/region and can be used in research for comparison of the myelin staining intensity between different groups (e.g., in demyelination studies).

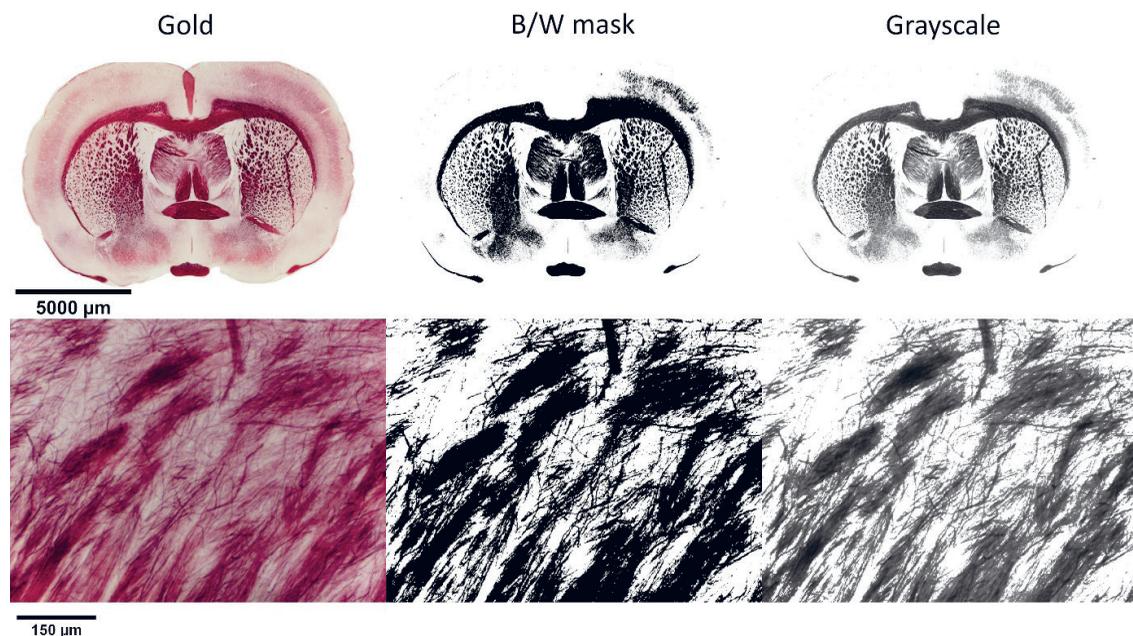
Gold staining protocol for nervous tissue is one of the methods of heavy metal impregnation that usually stains myelin sheath or neurofibrils. Many of the impregnation protocols suffer from unspecific photocatalytic deposition

of metal complexes, or metal ions arbitrarily react with structures within the nervous tissue. Our aims were to increase consistency and reproducibility of the protocol while obtaining the best possible staining, adequate for digital image analysis. This can be very useful when the neighboring sections are stained using (immuno)histochemical methods for localization of specific markers.

The key modifications we made to the existing protocol by Schmued were increasing section thickness from 25 to 35 micrometers, washing the sections with distilled



**Figure 4.** Different regions of brain with high axon density stained using gold impregnation, Nissl stain and MAP2 IHC. Represented regions: cingulum (cg), caudate putamen (CPu), habenula (Hb) and superficial gray layer of the superior colliculus (SuG). 10× objective, scale bar: 500 µm.



**Figure 5.** Representative images during the analysis process for measuring integrated density on the whole-brain sections and a sample under higher magnification (caudate putamen). Images represent photographs of gold impregnation-stained samples (Gold), black and white mask obtained using color thresholding in FIJI (B/W mask) and grayscale (8-bit) image of gold-stained structures obtained using black and white mask in FIJI (Grayscale) on both whole brain section (10× objective, scale bar: 5000 µm) and magnified sample (20× objective, scale bar: 150 µm).

water prior to incubation, performing the incubation in the humid chamber instead of glass jars, omitting the thiosulfate, working in a darkened (but not a completely dark) room, using only LED lights. With increased section thickness, free-float IHC was also attainable on the neighboring sections without section disintegration. More important, details in the cortex are better visualized, and

thickness of myelinated structures can be ascertained easier. Other modifications contribute to consistency and reproducibility. With this protocol, the reactions remain similar in intensity and reaction period from day to day. Because of this predictable reactivity it is possible to conduct longitudinal studies with little change in results, if any. In contrast to most of the impregnation protocols,

thiosulphate fixation is not necessary, but can be useful if thicker sections are used. We recommend abstaining from using too thin sections (less than 20 micrometers) because of mechanical stress imposed on the tissue during the preparation. Furthermore, thicker sections are generally recommended for methods involving substantial manipulation of the sections, such as free-floating IHC (18).

Traditional dyes for myelin staining in cryosections include osmium tetroxide, which can have harmful health effects, myelin fluorescence with 3,3'-diaminobenzidine (DAB), a well-known mutagen, palladium chloride, which is not useful for central nervous system tissue, Sudan family of dyes, that usually don't stain all types of lipids, and several other methods (19). Gallyash method can also be used to stain myelinated regions in the brain sections, but this method requires all chemicals and glassware to be ultrapure (purified from all contaminants). Gallyash is a multistep-multiresolution protocol and in each of the steps significant errors can occur when cryopreserved sections are used, and it is even more light-sensitive than gold staining (20,21).

Our modified and modernized gold impregnation method is an alternative that's on par with traditional methods and might be preferred due to excellent contrast and myelin color. During the testing of our method, all the images were suitable for digital analysis and quantification with very little (or none) background staining that would interfere with myelin isolation from images. Analysis of this type can easily show changes in architecture of myelinated structures and help in the investigation of demyelination processes and degenerations of the nervous tissue. Due to the ongoing research efforts in neuroscience, there is still a demand for new and improved methods for morphological studies of the brain. Our paper shows that impregnation methods can be used in modern neuroscience, coupled with contemporary image analysis software to obtain accurate, consistent, and reproducible results.

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