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Back to the future: improving storage of Golgi-stained mouse brain

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ABSTRACT

Morphological analysis of neuronal processes and their networks is a key aspect of neuroscience, with relevance from basic research to clinical practice due to the central role of neuronal development and plasticity in many neurological disorders. More than a century after its introduction, Golgi staining, a technique based on the random precipitation of metallic deposits in different neuronal subtypes, remains a highly valuable method for investigating the cellular morphology of neurons in the nervous system. Despite the wide range of protocols developed over the years, several limitations of the technique remain a matter of discussion. Among these is the need to extend sample preservation during the interval between staining and sectioning procedures without compromising the quality of the histochemical labeling. By adopting a specific processing method, the present study demonstrates that it is possible to embed murine nervous tissue following Golgi staining and to preserve the samples for extended periods prior to sectioning, while maintaining well-preserved and clearly detectable histochemical labeling across different regions and neurons of the mouse central nervous system.

Key words: Golgi staining; mouse brain; paraffin embedding.

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Ethics approval: the experimental procedures were performed in agreement with the European Council Directive 2010/63/EU on the care and use of laboratory animals, also following the guidelines established by the institution's animal welfare committee, the Ethics Committee of the University of Pavia (Ministry of Health, License number 935/2023-PR, approval date: 30 October 2023).

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Introduction

Also known as the "black reaction", the Golgi stain opened a new chapter in the history of nervous tissue histochemistry. Before its introduction, the two histochemical methods used to study nervous tissue, *i.e.* i) fixation, embedding, sectioning, and staining with hematoxylin or carmine, and ii) staining with chromic acid or potassium dichromate solutions followed by mechanical isolation of individual nerve cells, had major limitations. Although useful at the time and still valuable today for the morphological study of specific structures in other tissues and organs, the former does not yield optimal results in nervous tissue: it is unable to reveal the complete morphology of nerve cells and stains only the nucleus and small cytoplasmic portions of both small and large neurons. Conversely, chromic acid or potassium dichromate staining allowed tissue fixation and hardening for neuron isolation but was limited to large neurons and often broke terminal segments during the procedure.[1] To date, several methods have been developed to better study the morphology of individual cells of the nervous system, enabling even full three-dimensional reconstructions. Nevertheless, these techniques have limitations: dye distribution is uneven, fluorescence is often weak, and some methods may alter neuronal morphology, such as filling cells with carbocyanine dyes or staining *via* viral or biolistic gene transfer. Likewise, the use of transgenic mice does not represent an optimal solution, as it requires specialized equipment to visualize single cells and extensive breeding programs.[2]

In light of these modern limitations, the Golgi black reaction, developed by Camillo Golgi more than 150 years ago,[3] remains a powerful technique for clearly visualizing the morphology of glial and neuronal cells in the nervous system. It allows the identification of fine dendritic structures, such as spines, and their possible alterations following pharmacological treatments.[4]

The Golgi stain consists of impregnating nervous tissue with silver nitrate ($AgNO_3$) following treatment with potassium dichromate ($K_2Cr_2O_7$). This procedure results in red–black staining of neurons against a pale orange background, due to the formation of silver chromate salts (Ag_2CrO_4) or water-insoluble silver dichromate ($Ag_2Cr_2O_7$).[5-7] Since numerous physicochemical factors (*e.g.*, pH, temperature, salt conditions, developmental stage, species, impregnation time, and cellular metabolic activity) affect staining outcomes, the method eventually labels an apparently random population of cells. Over the years, several researchers have therefore sought to improve the original protocol by introducing new experimental procedures aimed at standardizing results and reducing limitations.[2]

Despite these efforts, certain limitations remain. For example, although Golgi-Kopsch staining often yields excellent results in formalin-fixed human samples, rapid Golgi or Golgi-Cox methods produce superior outcomes in freshly perfused mammalian brains.[8] Moreover, the literature reports degradation of stained sections, even after mounting.[9] Therefore, to overcome some of the limitations of the Golgi stain and its various modifications, the present study aimed to develop an experimental protocol that enables long-term preservation of histological preparations by combining the classical Golgi protocol with contemporary histological processing methods used for light microscopy.[3,10,11] This approach allows prolonged storage of nervous tissue samples from mice, which are among the most widely used *in vivo* preclinical models.

Materials and Methods

Mouse model employed

Wild-type (strain C57BL/6) male (n=5) mice (Charles River Italia, Calco, Italy) entered the Animal Care Facility of the University of Pavia at ~3 months of age and were acclimatized for three weeks before experiments, housed in humidity and temperature -controlled (50±10% with temperature at 21±2°C) vivaria (one animals/cage) and under a 12 h light/dark cycle throughout the experiments. Water and food were provided *ad libitum*.

Experimental procedures were performed in agreement with the European Council Directive 2010/63/EU on the care and use of laboratory animals, also following the guidelines established by the institution's animal welfare committee, the Ethics Committee of Pavia University (Ministry of Health, License number 935/2023-PR, approval date: 30 October 2023). All animals employed have been treated humanely, with due concern for distress and discomfort alleviation. This work is part of the project NODES, which has received funding from the MUR – M4C2 1.5 of PNRR funded by the European Union - NextGenerationEU (Grant agreement no. ECS00000036). After two months, mice were deeply anesthetized by isoflurane inhalation before decapitation (Aldrich, Milwaukee, WI, USA), and their brains were immediately removed and processed as described below.

Brain tissue fixation

Upon extraction, the cerebral hemispheres and the cerebellum were dissected by separating the two cerebral hemispheres and performing an incision at the level of the quadrigeminal tubercles to dissociate the cerebellum, thereby obtaining tissue samples approximately 3-4 mm in thickness. The samples were kept in 0.9% NaCl during the dissection procedure. Subsequently, the tissues were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 h.

Golgi stain and paraffin embedding

Once fixation was completed, the samples were rinsed in distilled water and immersed in a 3% potassium dichromate solution ($K_2Cr_2O_7$ in H_2O) for 3 days at room temperature and in the dark. At the end of this incubation period, the tissues were briefly rinsed in a 2% silver nitrate solution ($AgNO_3$ in H_2O) and subsequently incubated in 2% $AgNO_3$ for 3 days at room temperature and in the absence of light.

Once the staining process was completed, the samples were rapidly rinsed in 90% ethanol and then immersed in 90% ethanol for 1 hour. They were subsequently transferred to 100% ethanol for 15 min, rinsed in acetone, and incubated in acetone for 15 min. The samples were then moved to molten Paraplast X-TRA (Sigma Aldrich, Milan, Italy) and maintained at 60°C for 15 min, after which they were allowed to cool at room temperature. After 7 days, sixty-micrometers-thick sagittal sections of cerebrum and cerebellum were cut serially using a manual rotary microtome and collected on silane-coated slides. During sectioning, the tissue was intermittently moistened with 80% ethanol, and drops of the same solution were also used to allow complete flattening of the sections on glass slides. Once fully dried, the sections were mounted with a cover glass using Eukitt® (Sigma-Aldrich, St. Louis, MO, USA) and examined by bright-field microscopy.

Brightfield microscopy

All samples were examined using bright field microscopy, with Leica DM6B WF microscope (Leica microsystems,

Buccinasco, MI, Italy), and images were acquired with a Leica dfc 7000t CCD camera (Leica microsystems).

Results

In all examined samples, cells were stained dark brown to black, against a faint yellow–orange background, almost absent in some brain regions.

The analysis primarily focused on cells of the hippocampal formation, *i.e.*, dentate gyrus and Cornu Ammonis (CA), but also included various layers of the cerebral cortex, striatum, substantia nigra, and cerebellar cortex.

Figure 1 shows the results obtained after histochemical staining in different regions of the hippocampal formation. The staining clearly highlights the following structures, in dark brown to black tones on a pale orange background: the cell soma and processes of neurons in the subiculum (Figure 1a); the cell body, dendritic tree, and axon, including fine axonal collaterals, of pyramidal neurons in the CA1 and CA2 regions (Figure 1 b,c); the soma, dendritic arbor, and axon hillock of granule cells in the dentate gyrus, as well as the cell body and processes of mossy cells in the hilus (Figure 1 d,e); and the processes and cell soma of neurons in the CA3 region (Figure 1f).

The results of the histochemical reaction on cerebral cortex sections are shown in Figure 2. Histochemical staining in different cortical areas, i.e., the primary visual cortex (Figure 2a), primary somatosensory cortex (Figure 2b), and primary motor cortex (Figure 2c), highlights specifically the morphology of layer V pyramidal neurons. In these brain regions, background staining appears much fainter than previously observed in the hippocampal formation, with some areas showing an almost complete absence of background. The soma and dendritic processes of layer V pyramidal neurons in all examined cortical areas are well black-labeled, allowing the identification of the axon hillock in some neurons. In addition, some non-spiny neurons, likely basket or chandelier cells, are visible in the different cortical areas taken under examination.

The method herein employed also enables the detection of different cell types in subcortical brain regions, *i.e.*, the striatum and the substantia nigra, as well as at the cerebellar level. Specifically, distinct cellular populations are stained black/dark brown against a yellow/orange background (Figure 3). In the striatum, Medium Spiny Neurons (MSNs) are clearly identifiable, along with their dendrites and dendritic spines (Figure 3a); similarly, various cell types and cytoplasmic processes are prominently visible within the substantia nigra (Figure 3b). At the cerebellar level, staining is particularly evident in the cortical layers, where the soma and dendritic arborization of numerous Purkinje cells are clearly labeled. Additional cerebellar labeling is observed along climbing fibers, whose trajectory can be readily followed after staining, from the deep regions of the white matter to their terminal dendritic branching on Purkinje cells (Figure 3 c,d).

Discussion

Common histochemical stains used in neuroscience, such as Nissl staining, do not allow detailed visualization of the precise cellular morphology of the different neuronal populations within the nervous system. Conversely, more recent and sophisticated approaches, such as the use of specific antibodies in fluorescent

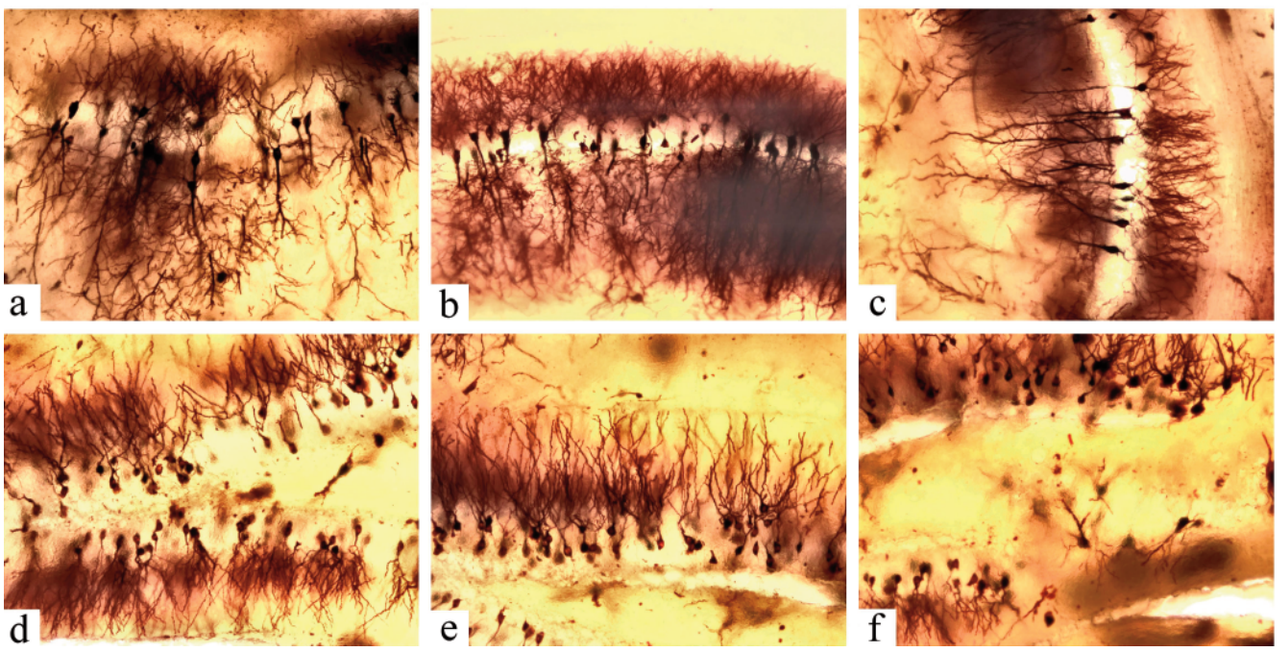

Figure 1. Representation of nervous cells in different regions of the mouse hippocampal formation, *i.e.*, subiculum (**a**), CA1 (**b**), CA2 (**c**), dentate gyrus (**d,e**), and CA3 (**f**) after Golgi staining of paraffin-embedded samples. Magnification: 40×.

immunohistochemistry and genetic labeling methods, enable a more accurate analysis of cellular morphology, albeit at the cost of substantially higher expenses and increased technical complexity.[12-14] By contrast, the Golgi method is a powerful, relatively simple and low-cost technique to study cell morphology, which involves sequential impregnation of tissue with potassium dichromate followed by immersion of the samples in silver nitrate to stochastically label a subset of neurons. This approach allows clear identification of all neuronal structures, from the cell soma to axonal projections and dendritic arbors.[15] The currently optimized Golgi staining methods use either fresh or fixed tissue samples; however, regardless of the protocol employed, whether standard Golgi, Golgi-Cox, or Golgi-Kopsch, following fixation, tissues must be processed and can only be preserved after sectioning.[3,15-17] This constraint precludes prolonged interruptions at intermediate stages of histological processing, as extended delays may compromise the staining outcome, potentially leading to partial or complete loss of histochemical labeling in neurons or specific regions of the nervous system. To overcome this limitation, the present study shows for the first time that a specific paraformaldehyde fixation protocol, followed by rapid dehydration and clearing, allows paraffin embedding and long-term storage while preserving neuronal labeling throughout the adult mouse central nervous system. Using the proposed modified Golgi protocol, distinct neuronal populations in the cerebral cortex (primary visual, somatosensory, and motor areas), hippocampus, subcortical regions (striatum and

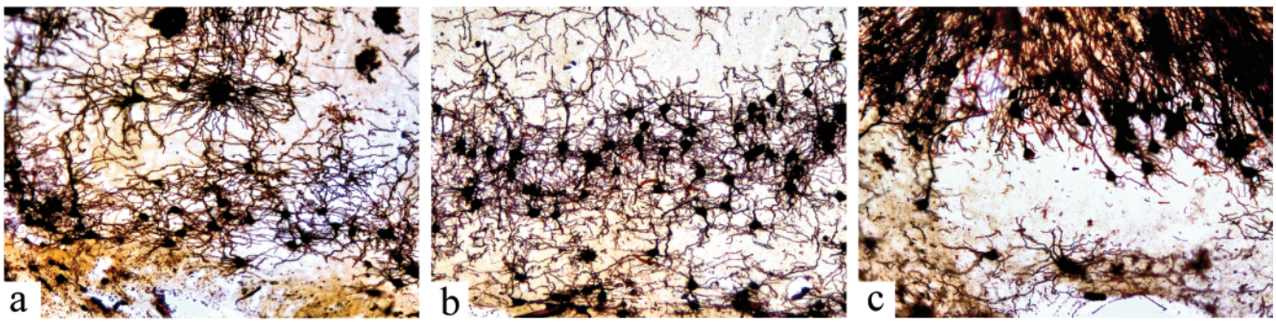

Figure 2. Representation of neurons from the internal pyramidal layer (layer V) of different regions of the mouse cerebral cortex, namely primary visual cortex (**a**), primary somatosensory cortex (**b**), and primary motor cortex (**c**), following Golgi staining of paraffin-embedded samples. Magnification: 40×.

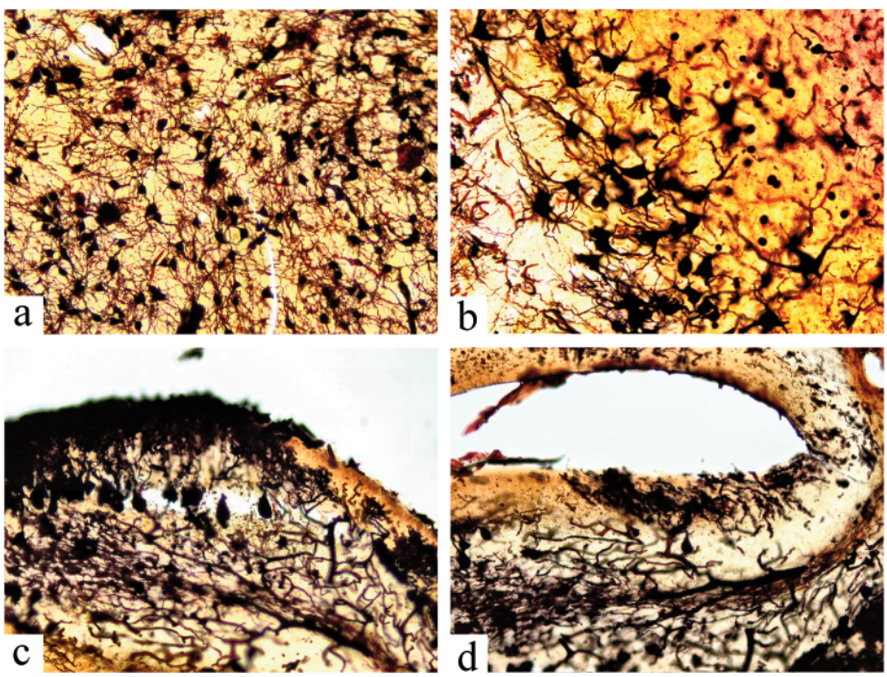

Figure 3. Representation of neuronal cells of the striatum (**a**), substantia nigra (**b**), and cerebellar cortex: superficial (**c**) and deep (**d**) cortical areas of the lobules in Golgi-stained, paraffin-embedded brain samples. Magnification: 40×.

substantia nigra), and cerebellar cortex exhibit intense labeling clearly visible against the pale yellow-orange background. The staining highlights the finest neuronal morphological structures typically revealed by Golgi methods[3] across all examined regions, including neuronal somas, dendritic arbors, axonal projections such as fine branches of layer V pyramidal neurons or cerebellar climbing fibers, and even the smallest dendritic spine protrusions of medium spiny neurons (MSNs) in the striatum. However, although various neuronal populations in different brain regions are well-stained and easily identifiable, allowing for a detailed study of neuronal morphology, some regions appear more amenable to the proposed protocol, with selected neuron types exhibiting stronger labeling in specific cellular compartments. Indeed, in the cerebral cortex the staining preferentially highlights layer V pyramidal neurons, particularly emphasizing their dendritic arborizations, which are also evident in granule neurons of the dentate gyrus and Purkinje cells in the cerebellum.[18-20] Moreover, not all neuronal populations were clearly identifiable. For example, in the cerebral cortex, only certain non-spiny neurons (likely basket or chandelier cells) were visible, whereas in the cerebellum no detectable labeling of granule cells, or other neuronal populations such as Golgi cells, was observed.[18,21] In the striatum, broad neuronal populations and fine membrane specializations, including dendritic spines,[22] can be visualized, while axonal projections, collaterals, and fine dendritic branches of pyramidal neurons can be traced in the substantia nigra, Ammon's horn, and subiculum.[23,24] Noteworthy is also the variation in the background signal observed across the different brain regions under study. Although an orange-hued background is present in the deep brain areas, as well as in various hippocampal regions and in the cerebellar cortex, this background signal decreases significantly when assessing the distinct cortical areas of the cerebral cortex. This difference in background signal may be due to the greater exposure of superficial regions of the nervous system to ethanol, which can enhance staining quality, as in the case of Golgi-Cox, leading to partial solubilization of the silver chromate crystals.[25] However, this does not fully explain why the same does not apply to the examined cerebellar regions and therefore warrants further detailed studies; a possible solution could be the slight adjustment of dehydration times to find the optimal balance between reducing background signal across all areas of the nervous system while preserving neuronal staining.

Although invented over a century and a half ago, Golgi staining remains an extremely reliable histochemical method for studying neuronal morphology and the cytoarchitecture of the nervous system. However, this method presents some limitations that still require careful attention and potential optimization, such as reducing non-specific staining of neurons. Unlike previous protocols, the present study demonstrates that it is possible to perform silver impregnation on mouse nervous tissue, achieving intense and clearly identifiable labeling of the finest neuronal structures across various regions of the murine nervous system after paraffin embedding and storage. This approach thus allows easier long-term preservation of samples without compromising the efficiency or the valuable morphological and cytoarchitectural information provided by this historical staining technique.

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