Int. J. Morphol.,
43(6):1869-1878, 2025.

Adaptation of the Mailland Technique for Neuroanatomical Staining: A Cost-Effective and Accessible Method for Teaching Brain Structure

Adaptación de la Técnica de Mailland para la Tinción Neuroanatómica: Un Método Económico y Accesible para la Enseñanza de la Estructura Cerebral

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PRADO-SANHUEZA, A.; VEUTHEY, C.; VARGAS, D.; ROA, I.; LIZANA, P.A.; MASUKO, T. & OTTONE, N.E. Adaptation of the Mailland technique for neuroanatomical staining: a cost-effective and accessible method for teaching brain structure. *Int. J. Morphol., 43(6)*:1869-1878, 2025.

SUMMARY: Effective visualization of central nervous system structures is essential for neuroanatomical education, particularly at the undergraduate level where access to fresh specimens and advanced imaging resources is often limited. Classical staining methods such as those developed by Sincke, Mulligan, and Braak have contributed significantly to anatomical teaching; however, many of these protocols involve complex procedures, hazardous chemicals, or prolonged fixation and processing times that hinder their widespread application in educational contexts. In response to these limitations, this study aimed to develop and evaluate a simplified, low-toxicity adaptation of the Mailland technique for staining formalin-fixed brain tissue. Two bovine (Bos taurus) brains were used, sectioned parasagittally into 18 slices of 3 mm thickness, and stained with ferric chloride and potassium ferrocyanide at varying concentrations (1%, 0.75%, and 0.5%) and immersion times. The resulting blue pigmentation allowed clear differentiation between gray and white matter, with staining intensity increasing proportionally with concentration and exposure time. Group 1 (1% for 5 min and 1 min, respectively) exhibited the most intense coloration, while Group 6 (0.5% for 1 min and 30 s) still demonstrated a reliable contrast, highlighting the method's efficiency even under minimal conditions. The adapted protocol requires no specialized equipment, operates at room temperature, and uses readily available reagents, making it highly accessible for academic laboratories with limited infrastructure. Compared to other classical methods, our approach is faster, more economical, and safer, with consistent results across slices. This technique, therefore, provides a valuable pedagogical tool for enhancing neuroanatomical education through improved structural visualization.

KEY WORDS: Brain staining; Mailland technique; Neuroanatomy; Anatomical education.

INTRODUCTION

Anatomical techniques for the preservation and study of tissues constitute a valuable tool in preparation for medical education (Skopnik-Chicago *et al*., 2021), particularly highlighting their usefulness in the teaching of neuroanatomy (Ccorahua-Rios *et al*., 2020; Villegas-Gomez *et al*., 2023). In recent years, the need to enhance neuroanatomy education at the undergraduate level, and to ensure its effective integration with clinical disciplines such as neurology and neurosurgery, has driven the development of multiple anatomical approaches for working with brain structures while preserving their morphological characteristics (Villegas-Gomez *et al*., 2023). Among these, neurostaining techniques have emerged as practical and engaging methods that promote active learning and spatial understanding.

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Received: 2025-06-22 Accepted: 2025-08-16

Depending on the specific objectives of observation or study, various staining protocols have been developed to differentiate neuronal components. For example, immersing brain structure sections in a series of chemical solutions (Forlizzi *et al*., 2020; Ccorahua-Rios *et al*., 2020) facilitates rapid and clear macroscopic identification of the substances that compose gray and white matter (Loftspring *et al*., 2008). This proves especially useful not only for the study of normal brain anatomy but also for visualizing pathological changes in the nervous system.

Since the early 20th century, several methods have been developed for staining and preserving cadaver brain sections to enhance macroscopic neuroanatomy education. Among them, the Prussian blue-based protocol described by Barnard, Roberts, and Brown (1949) provides a simple yet effective method to differentially stain gray and white matter, while also embedding the sections in butyl methacrylate plastic for prolonged use. This approach results in brilliant and permanent color contrast, improves specimen durability, and facilitates student interaction by reducing unpleasant handling characteristics. Later refinements by Roberts and Hanaway (1969) introduced the copper sulfate–phenol–ferrocyanide method, which imparts a red-brown color to gray matter while leaving white matter unstained. Their improvements included formalin perfusion for fixation, machine slicing into uniform 4 mm sections, and optimized staining and washing steps, resulting in specimens with excellent contrast that closely resemble freshly cut brain tissue. These protocols underscore the enduring relevance of gross staining techniques for creating long-lasting, high-quality neuroanatomical teaching materials.

The importance of these neurotechniques becomes even more evident when considering the fragility of brain tissue, which can easily deteriorate due to environmental factors or improper handling. After staining, specimens are susceptible to fading when exposed to sunlight, while inadequate preservation, such as prolonged storage without proper fixatives, can lead to dehydration and deformation. Therefore, combining effective staining with preservation strategies is essential for maintaining the morphological integrity of samples over time.

Numerous staining methods have been documented, including those proposed by Mulligan, Mailland, Barnard, Robert and Brown, and Green, each contributing valuable innovations to neuroanatomical preparation (Rodrigues, 2010; Merini *et al*., 2014). These techniques allow for clearer visualization and mapping of both cortical and deep brain structures (Azu *et al*., 2019), thereby facilitating the identification of early neuropathological changes when used alongside modern imaging technologies.

In this context, a thorough understanding of normal brain morphology remains foundational. Access to high-quality stained anatomical specimens enhances the ability of students in the health sciences to recognize and interpret both healthy and pathological brain anatomy with greater precision.

Early macroscopic staining methods, such as the Prussian blue reaction described by Sincke in 1926, relied on the immersion of formalin-fixed brain slices in ferric chloride followed by potassium ferrocyanide, resulting in selective blue staining of gray matter. While initially attributed to a specific chemical affinity for iron, Blair *et al*. (1932), demonstrated that this differentiation is primarily governed by physical factors, including differential reagent penetration and adsorption between gray and white matter. This understanding broadened the range of applicable staining techniques and emphasized the role of tissue architecture in macroscopic contrast.

The aim of this study was to develop an anatomical brain staining technique that reduces toxic effects, optimizes implementation time and supply costs, and ensures proper preservation for the generation of neurological study materials.

MATERIAL AND METHOD

Two bovine (*Bos taurus*) brains were extracted from previously frozen cadaveric material, which underwent a supervised thawing process over 72 hours. After this period, a preliminary fixation of the brain was performed by injecting 10 % formalin retrobulbarly in a bilateral, dorsal, ventral, lateral, and medial manner. This approach aimed to infiltrate the intraorbital foramina and thereby fix the cranioventral portion of each brain. Additionally, to fix the caudal portion of the brain, 10 % formalin was injected through the foramen magnum, ensuring the solution covered as much tissue as possible by injecting the necessary volume until the solution overflowed from the cranial cavities. Following the formalin infiltrations, the skin and all soft tissues covering the bony structure were removed. For this procedure, standard dissection surgical instruments were used on a surgical table. Once the process was completed, the brains were submerged in a 20 % formalin solution for one week to ensure proper fixation and to preserve their original morphology.

After the fixation period, the specimen was rinsed with running water, and the brain was subsequently extracted. On a table equipped with a press and using a cutting grinder, three cuts were made in the skull to expose the brain. Carefully, bony structures of the neurocranium were removed until reaching the dura mater. Using blunt dissection,

superficial fixation elements were removed without damaging brain tissue. Once the area was cleared, the brain was gently lifted, and blunt dissection was completed on the remaining connections. The brain was then carefully extracted to avoid any damage. After extraction, the brain was submerged in 20 % formalin for one month. Following this period, the concentration was reduced to 10 % formalin for an additional three months.

After the fixation period, the brains were rinsed with running water for 24 hours. Longitudinal sectioning of the specimens was then performed using a circular saw lubricatedwith liquid soap to facilitate the cuts. This saw is commercially available as the deli slicer Bozzo, model: HBS-300[a]. During the procedure, 18 parasagittal sections were obtained, each measuring 3 mm in thickness.

Subsequently, the obtained slices were rinsed with running water for 1 hour inside a tray, ensuring that the water did not directly contact the tissue in order to avoid mechanical damage during washing. The slices were then divided into six groups, each consisting of three consecutive sections. These six groups were established to allow for modifications of the Mailland technique, specifically regarding exposure times and reagent concentrations. A detailed summary of the experimental protocol, from tissue preparation to staining, is presented in Table I.

Originally, the Mailland method consists of successively immersing nervous tissue slices in ferric chloride (FeCl) (2 %) and potassium ferrocyanide (K[Fe(CN)]) (2 %) solutions, with the goal of producing a blue coloration in the sample, thereby enabling the differentiation between white matter and gray matter. After this step, the slices are submerged in nitric acid (1 %) for 24 hours (Mailland, 1926), in order to achieve greater durability compared to slices fixed only in 10 % formalin.

For this study, 350 ml of each reagent, ferric chloride and potassium ferrocyanide, were prepared using distilled water at concentrations of 1 %, 0.75 %, and 0.5 %. The solutions were placed in properly labeled containers of optimal size to accommodate the different brain slices (Fig. 1).

The six groups of slices were immersed in the corresponding reagent concentrations, with varying immersion times (Table II; Fig. 2). First, the slices were submerged in the ferric chloride solution, followed by rinsing with running water and then distilled water, allowing them to drain for a few seconds. Second, the slices were immersed in the potassium

Table I. Overview of the experimental methodology applied to bovine brain plastination and histochemical contrast using the Mailland technique.

Step	Procedure	Details
1. Specimen acquisition	Bovine brains	Two (*Bos taurus*) brains from previously frozen cadavers.
2. Thawing process	Supervised thawing	72 hours at room temperature.
3. Preliminary fixation	Retrobulbar and foramen magnum injection	10 % formalin injected bilaterally (dorsal, ventral, lateral, medial) to fix cranioventral and caudal brain regions.
4. Soft tissue removal	Standard dissection	Skin and soft tissues removed to expose skull using surgical instruments.
5. Immersion fixation	20 % formalin immersion	One week to ensure deep and uniform fixation.
6. Brain extraction	Craniotomy and blunt dissection	Three cuts made; neurocranial bones removed to extract brain intact.
7. Post-extraction fixation	20 % and 10 % formalin immersion	20 % formalin for 1 month, then 10% formalin for 3 months.
8. Rinsing	Running water	24-hour rinse after fixation period.
9. Sectioning	Longitudinal parasagittal cuts	18 slices (3 mm thick) using circular saw (Bozzo HBS-300[a]) lubricated with liquid soap.
10. Pre-treatment rinse	Water rinse in tray	1-hour rinse without direct contact to tissue.
11. Grouping	Six experimental groups	Each group with 3 consecutive slices; assigned different reagent concentrations and exposure times.
12. Histochemical contrast (Modified Mailland method)	Iron-based staining	Immersion in $FeCl_3$ followed by $K_4[Fe(CN)_6]$, with concentrations of 1 %, 0.75 %, and 0.5 %; sequential rinses with water and storage in 1% nitric acid (24 h).
13. Final preservation	Storage in 10% formalin	All slices stored in labeled containers after staining and acid treatment.

Fig. 1. Representative appearance of the reagents used for brain tissue staining. A. Ferric chloride solution, characterized by its yellow coloration. B. Potassium ferrocyanide solution, displaying a pale tone. Both reagents are shown at different concentrations: *1 % (single asterisk), **0.75 % (double asterisks), and **0.5 % (triple asterisks).

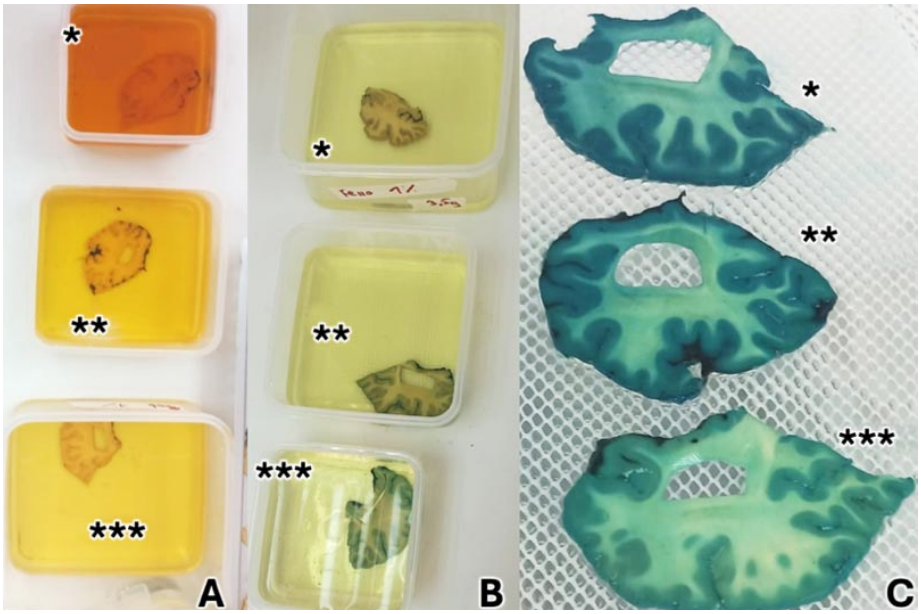

Fig. 2. Macroscopic appearance of bovine brain (Bos taurus) slices at different stages of the staining process using the modified Mailland technique. A. Brain slices immersed in ferric chloride solution. B. Brain slices after immersion in potassium ferrocyanide solution, followed by rinsing with running water and distilled water. C. Brain slices after exposure to both reagents, showing different staining intensities according to concentration: *1 % (single asterisk), **0.75 % (double asterisks), and **0.5 % (triple asterisks).

Table II. Exposure times (in minutes and seconds) of brain (Bos taurus) tissue slices to ferric chloride and potassium ferrocyanide solutions, across six experimental groups. Columns indicate reagent concentration (1 %, 0.75 %, and 0.5 %) and rows corresponding to each group. All samples were processed following a modified Mailland staining protocol, with variations in reagent concentration and immersion time to assess staining intensity.

Group	**Ferric chloride**			**Potassium ferrocyanide**		
	1 %	0.75 %	0.5 %	1 %	0.75 %	0.5 %
1	05:00 min	05:00 min	05:00 min	01:00 min	01:00 min	01:00 min
2	05:00 min	05:00 min	05:00 min	00:30 s	00:30 s	00:30 s
3	02:30 min	02:30 min	02:30 min	01:00 min	01:00 min	01:00 min
4	02:30 min	02:30 min	02:30 min	00:30 s	00:30 s	00:30 s
5	01:00 min	01:00 min	01:00 min	01:00 min	01:00 min	01:00 min
6	01:00 min	01:00 min	01:00 min	00:30 s	00:30 s	00:30 s

ferrocyanide solution, and then rinsed again with running water followed by distilled water, allowed to drain briefly, and finally stored in nitric acid for 24 hours. After this period, the slices were stored in containers filled with 10 % formalin.

RESULTS

Eighteen sagittal slices of bovine brain were obtained, each 3 mm thick. When grouping the slices, every three consecutive sections were selected to ensure similarity within each group and to facilitate the observation of differences resulting from the methodology applied. The preparation of the reagents at different concentrations (1 %, 0.75 %, 0.5 %) resulted in variations in staining intensity, as shown in Figure 2.

The six groups of samples, which were processed using different reagent concentrations and exposure times, revealed distinct differences in the staining intensity of the brain, cerebellum, and brainstem. Longer exposure times to 1 % ferric chloride and potassium ferrocyanide solutions produced more intense staining. In contrast, slices exposed to lower concentrations (0.5 %) and shorter immersion times exhibited lighter staining, as shown in Figure 3.

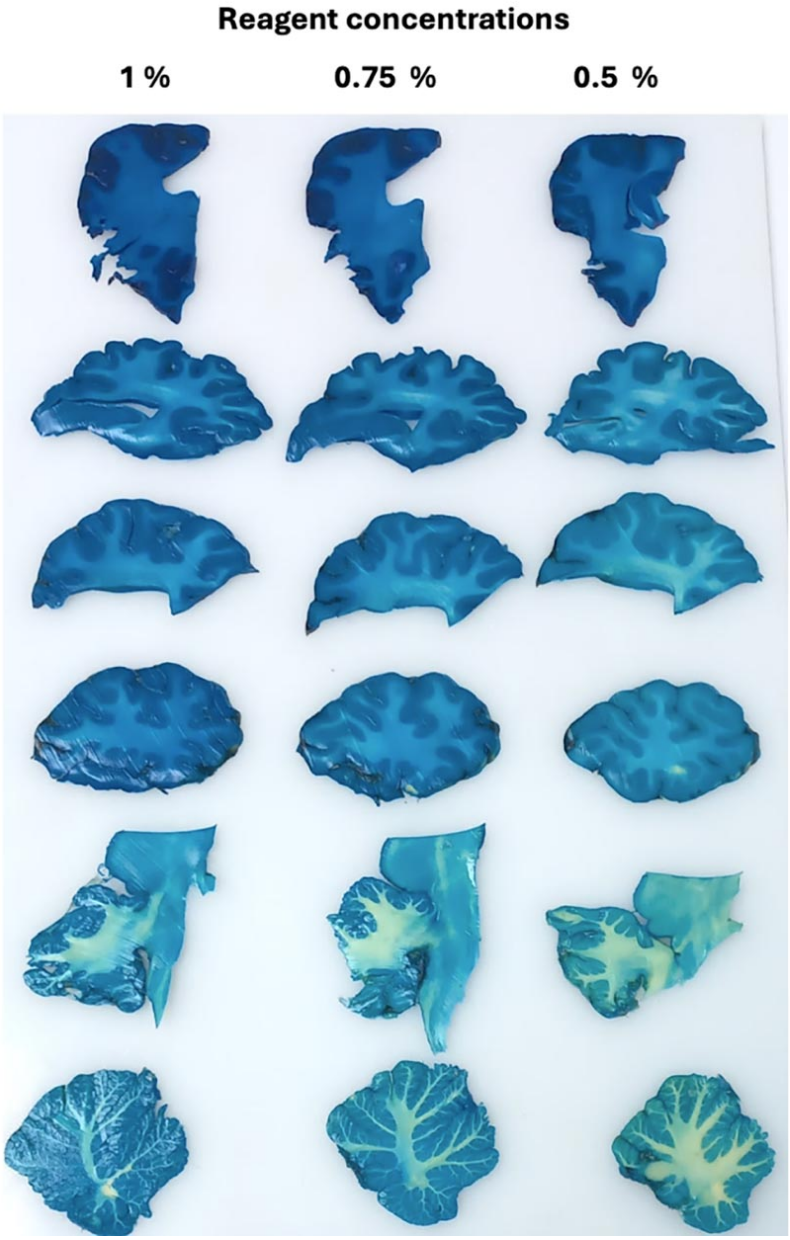

Fig. 3. Bovine brain (Bos taurus) slices arranged in six groups, each consisting of three consecutive sections. Columns represent different reagent concentrations: 1 %, 0.75 %, and 0.5 %. Rows correspond to Groups 1 through 6, each defined by specific exposure times to ferric chloride and potassium ferrocyanide solutions as follows:

Group 1: 5 min ferric chloride, 1 min potassium ferrocyanide;

Group 2: 5 min ferric chloride, 30 s potassium ferrocyanide;

Group 3: 2.5 min ferric chloride, 1 min potassium ferrocyanide;

Group 4: 2.5 min ferric chloride, 30 s potassium ferrocyanide;

Group 5: 1 min ferric chloride, 1 min potassium ferrocyanide;

Group 6: 1 min ferric chloride, 30 s potassium ferrocyanide. This layout allows comparative evaluation of staining intensity according to concentration and exposure time.

Based on these findings, the methodology used for Group 6 was repeated with additional consecutive sagittal brain slices to evaluate its effectiveness on other types of sections. As illustrated in Figure 4, this resulted in optimal tissue staining, with clearly differentiated structures (white matter and gray matter) based on the intensity of the acquired pigmentation.

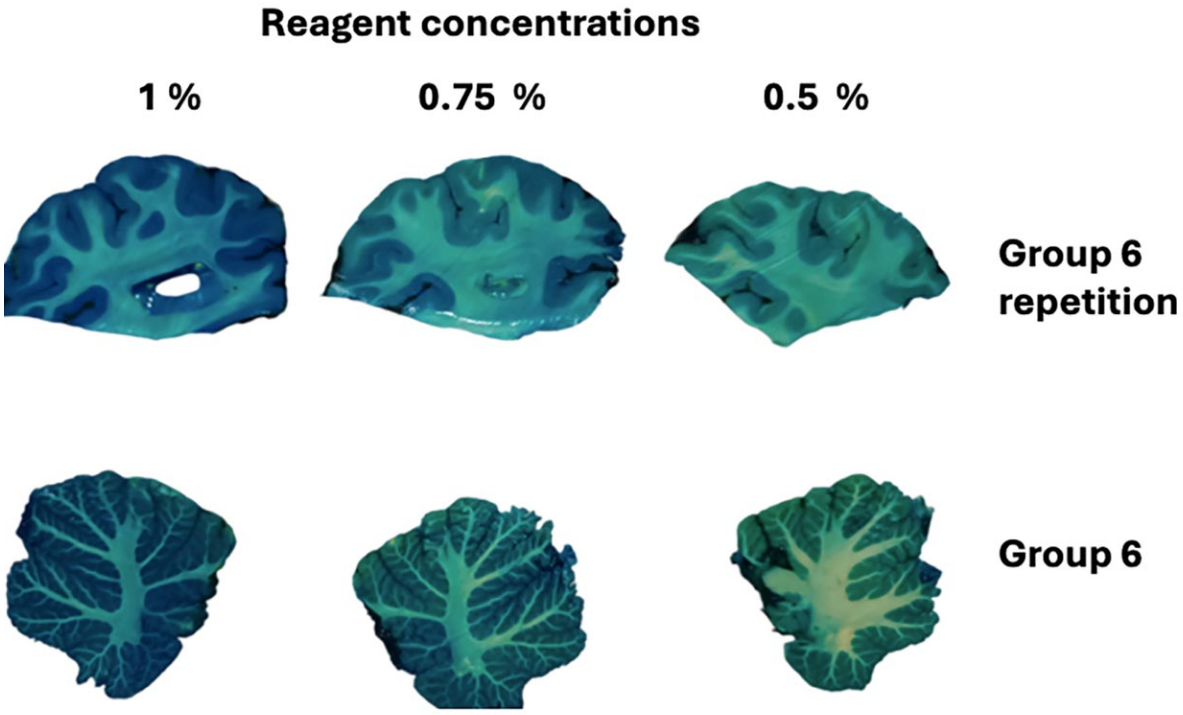

Fig. 4. Brain (Bos taurus) slices arranged in two groups, each consisting of three consecutive sections. Columns represent different reagent concentrations: 1 %, 0.75 %, and 0.5 %, while rows are organized by experimental groups. The first row shows a repetition of the Group 6 methodology (1 minute in ferric chloride and 30 seconds in potassium ferrocyanide), applied to consecutive sagittal brain slices. The second row displays the same staining protocol applied to different types of sections, in order to compare staining consistency across various anatomical planes.

DISCUSSION

The progressive evolution of techniques for studying brain structures has been notable since Sincke, whose simple two-reagent method was later improved by Mailland. Le Gros Clark further analyzed the staining process, focusing on the molecular composition of neuronal cells, which ultimately allowed Mulligan to develop his own technique. The Mulligan technique is a complex method involving multiple steps. In his publication, Mulligan (1931) noted that Le Gros Clark, in 1930, observed that when using the Sincke staining technique, it might be possible for the brain's white matter not to stain, as he had observed unstained sections of it. He hypothesized that the lipids composing the myelin sheath of the white matter extended throughout the adjacent brain tissue, protecting the axonal cylinders and neuroglia from the action of the reagents. This led Mulligan to propose the use of a fat-soluble solvent and to describe his technique, which involves the preparation of the "Mulligan solution" (Phenol 4.0 g; Hydrochloric acid 0.125 ml; Copper sulfate 0.5 g; Water 100 ml). Brain slices are immersed in this solution at a temperature of 60–65 °C, followed by rinsing and immersion in a tannic acid solution (2%). After another rinse, the slices are submerged in an iron-alum solution (0.5%), and finally fixed in 70° ethanol (Meneses *et al*., 2004). Additionally, Mulligan explained that the Mailland technique is derived from Sincke's method from 1926 (Mulligan, 1931), in which, after fixation of brain tissue in formalin, slices were immersed in an aqueous solution of ferric chloride (2%), followed by rinsing and immersion in an aqueous solution of potassium ferrocyanide (2%). This resulted in a dark blue staining of gray matter and a pale blue staining of white matter (Mainland, 1926; Meneses *et al*., 2004).

In this context, Roberts and Hanaway (1969) proposed a technical modification of Mulligan's method that includes improvements in the fixation, sectioning, and

staining steps. In their protocol, initial perfusion with 40% formalin followed by immersion in 10% formalin for 2 to 4 weeks ensures better tissue fixation. Subsequently, 4 mm sections are obtained using an electric rotary-blade slicer, optimizing the uniformity of the cuts. For staining, they replaced the use of iron alum and tannic acid with a CuSO-phenol solution at 60 °C for 6 minutes, followed by immersion in a 2 % potassium ferrocyanide solution, resulting in an intense reddish-brown color in the gray matter with clear differentiation from the white matter. Additionally, they significantly reduced washing times and simplified the handling of the slices without compromising the quality of morphological contrast. These modifications stand out for their effectiveness and reproducibility, offering an intermediate alternative between complex techniques and more accessible methods such as Mailland's.

Another notable approach was proposed by Braak (1978), who developed a simplified and durable staining technique using copper phthalocyanine dye (Astra blue) for block-staining thick formalin-fixed human brain slices. Unlike Mulligan's method, which relies on superficial staining and requires careful handling, Braak's technique impregnates the entire tissue, producing a vivid blue contrast in gray matter with long-term stability. The procedure, which includes oxidation with performic acid and immersion in Astra blue solution, is less technically demanding, avoids hazardous heating or complex reagent handling, and enables manipulation of the stained slices without risk of fading or damage. These features make it particularly suitable for educational settings and long-term teaching collections.

Blair *et al*. (1932), confirmed that variations in staining intensity between gray and white matter are largely attributable to tissue permeability and surface adsorption. In their experiments, even mechanical abrasion of white matter induced increased staining; supporting the hypothesis that differential physical access of reagents underlies the observed color contrast. This interpretation was further validated by their use of non-iron-based reactions, which produced equally effective differentiation, highlighting the limited role of intrinsic iron content in fixed tissue.

Compared to our findings, which demonstrate that minimal concentrations and short exposure times are sufficient to achieve clear differentiation of gray matter, the protocol proposed by Roberts and Hanaway (1969) relies on higher reagent volumes, longer pre-processing times (including perfusion and extended formalin immersion), and a different chromatic outcome, reddish-brown rather than blue, highlighting how distinct methodological decisions can lead to similar goals in terms of anatomical contrast, albeit with varying degrees of complexity, cost, and accessibility. Similarly, Braak (1978) introduced a block-staining method using the copper phthalocyanine dye Astra blue, which enables uniform, non-fading staining of thick brain slices through deep tissue impregnation. While our approach offers a rapid, tunable method with minimal handling and chemical risk, Braak's technique emphasizes simplicity, long-term stability, and resilience to physical manipulation, though it requires prolonged staining times and does not allow modulation of stain intensity. Taken together, these comparisons illustrate the spectrum of neurostaining methodologies, ranging from highly controllable surface staining to durable bulk staining, each with advantages depending on intended use, whether for experimental flexibility or long-term educational deployment.

A comparative summary of the main macroscopic brain staining techniques described in the literature is presented in Table III. This comparison highlights key differences across protocols, including reagent types, fixation methods, target tissue effects, and methodological complexity. Our adapted version of the Mailland technique stands out for its simplicity, reproducibility, and low toxicity, while still achieving effective contrast between gray and white matter. Unlike other approaches that require multi-step procedures, elevated temperatures, or hazardous chemicals, our method utilizes minimal concentrations and short exposure times, making it suitable for academic settings with limited resources.

In our study, a series of stains were obtained with varying degrees of blue intensity in tissues containing gray matter, as shown in Figure 3. This variation is attributed to the two factors manipulated during the procedure: concentration and exposure time. At the lowest concentration (0.5%) and shortest exposure time (1 minute for ferric chloride and 30 seconds for potassium ferrocyanide), the resulting blue tone was the lightest within the spectrum observed, represented by Group 6, as seen in Figures 2 and 3. Conversely, as the concentration and exposure time increased, the tissue pigmentation became more intense. This is particularly evident at the highest concentration (1%) and the longest exposure time (5 minutes for ferric chloride and 1 minute for potassium ferrocyanide), corresponding to Group 1, which is shown in Figure 2.

The progressive evolution of techniques for studying brain structures has been notable since Sincke, whose simple two-reagent method was later improved by Mailland. Le Gros Clark further analyzed the staining process, focusing on the molecular composition of neuronal cells, which ultimately allowed Mulligan to develop his own technique. However, Mulligan's method requires the preparation of solutions with multiple reagents, including toxic substances

Table III. Comparative overview of classical and contemporary macroscopic brain staining techniques, including the method proposed in the present study.

Author(s)	Staining Reagents	Target Tissue	Fixation Method	Key Features
Sincke (1926)	Ferric chloride + Potassium ferrocyanide	Gray matter (dark blue), white matter (unstained)	Formalin	First systematic method using iron salts; simple
Mailland (1926)	Same as Sincke: $FeCl_3$ + $K_4[Fe(CN)_6]$	Same as Sincke	Formalin	Optimized version of Sincke; less toxic
Mulligan (1931)	Phenol-HCl-$CuSO_4$+ Tannic acid + Iron alum	Gray matter (reddish-brown), white matter (unstained)	Formalin + 60–65 °C treatment	Multi-step, high-temp, complex; intense color
Blair *et al*., (1932)	Multiple: e.g., Na_2S + $Co(NO_3)_2$; Starch + Iodine	Gray matter (varied colors), white matter minimally stained	Formalin	Physical staining mechanism; variety of reactants tested
LeMasurier (1935)	Phenol pretreatment + Prussian blue	Gray matter (intensified), white matter protected	Formalin	Durable staining; phenol improves selectivity
Roberts & Hanaway (1969)	$CuSO_4$ + Phenol + $K_4[Fe(CN)_6]$	Gray matter (reddish-brown), white matter (unstained)	Perfusion (40 %) + immersion (10 %) formalin	Improved sectioning, reduced wash, reproducible
Braak (1978)	Astra blue (Copper phthalocyanine dye)	Gray matter (vivid blue), full tissue impregnation	Formalin	Stable, deep staining; long-term preservation
Alston (1981)	$CuSO_4$-Phenol-HCl + Xylene/Polyclens + NaOH + $K_4[Fe(CN)_6]$	Gray matter (brick red), white matter (brilliant white)	Formalin-fixed + gelatin embedding	Batch staining; image analysis-compatible; low toxicity; excellent contrast; color-stable for 5+ years
Suriyaprapadilok & Withyachumnarnkul (1997)	Mulligan, Le Masurier, Roberta, Braak y Alston methods	Gray matter (variable: gray-black, brilliant blue, reddish brown, bluish green, brick red), white matter unstained	Formalin (thick slices: 4–6 mm)	Comparison of five techniques for plastination preparation; all showed intense coloration of gray matter; shrinkage was less than 1 % after staining (except for Braak: 2 %), and approximately 10 % after plastination
Wu & Kiernan (2001)	Phenol pretreatment + Copper phthalocyanine (CPTS 1 %)	Gray matter (bright blue), white matter protected	Formalin (4 %) for 6–24 months	Three-step protocol; stable, surface-specific staining; compatible with plastination; no fading after 4 years
Azu *et al*., (2019)	Mulligan technique	Gray-white differentiation, cortical thickness 3-5 mm	5 mm Formalin slices	Morphometric study; rightward asymmetry, good cortical delineation
Forlizzi *et al*., (2020)	Mulligan solution (Phenol + $CuSO_4$ + HCl) + $FeCl_3$ + $K_4[Fe(CN)_6]$	Gray matter (intense blue), white matter (unstained)	10 % formalin for 30 days	Standardized Mulligan protocol; emphasizes meningeal removal and cold water shock; highly detailed CNS staining for teaching
Our study	Ferric chloride + Potassium ferrocyanide (0.5–1%)	Gray matter (blue), white matter (faint or unstained)	Stepwise fixation: 10 % + 20 % formalin (1–3 months)	Adapted Mailland protocol; tested multiple concentrations and times; low-toxicity optimized for educational use

that demand additional equipment such as fume extractors, as well as thermostatically controlled ovens or water baths to reach the required temperatures, along with biosafety measures.

In this historical context, LeMasurier (1935) introduced a simplified and durable approach by combining Mulligan's phenol pretreatment with the Prussian blue sequence. His method produced brilliant and lasting contrast

between gray and white matter, with staining stability confirmed even after prolonged exposure to light and alcohol. Phenol was shown to act as a protective barrier for white matter, improving the selectivity of the stain. These features made the protocol highly suitable for educational and museum applications, while reducing the complexity associated with earlier methods.

For this reason, we believe the Mailland technique offers greater accessibility for universities seeking to conduct research while also producing teaching materials for health science students. Compared to the previously described methodologies mentioned in this study, Mailland's technique stands out for requiring less time and fewer steps to achieve visible results. As described in the methods section, only two reagents are needed to achieve tissue staining, along with an acid solution to preserve the pigmentation. This can be performed at room temperature.

Moreover, as shown in our results, the minimum tested exposure time, 1 minute in ferric chloride solution and 30 seconds in potassium ferrocyanide, both at 0.5 % concentration, was sufficient to visualize gray matter staining. In contrast, other techniques such as those by Barnard, Robert and Brown, Green, or the Mulligan method, require the preparation of complex solutions (e.g., Mulligan solution) or a series of solutions, many of which involve immersing tissue in acidic solutions. These also require exposing the tissue to temperatures around 60°C, which increases the risk associated with handling toxic substances without appropriate equipment like air extractors. This results in higher costs due to necessary infrastructure and reagents, and leads to longer delays in obtaining the expected outcomes.

CONCLUSION

The adapted Mailland staining protocol presented in this study proved to be a simple, cost-effective, and low-toxicity method for differentiating gray and white matter in formalin-fixed brain tissue. The technique demonstrated reliable and reproducible results across multiple slices, even when using minimal reagent concentrations and short exposure times. Unlike more complex historical methods, this approach requires no special equipment, is safe for educational use, and provides clear structural contrast suitable for neuroanatomical instruction. Furthermore, when compared to a wide range of classical and modern protocols (as detailed in Table III), the present method offers a compelling balance between efficacy, accessibility, and safety. These characteristics make it a valuable pedagogical tool, particularly in academic environments with limited infrastructure, and support its broader application in both teaching and research settings.

PRADO-SANHUEZA, A.; VEUTHEY, C.; VARGAS, D.; ROA, I.; LIZANA, P.A.; MASUKO, T. & OTTONE, N.E. Adaptación de la técnica de Mailland para la tinción neuroanatómica: Un método económico y accesible para la enseñanza de la estructura cerebral. *Int. J. Morphol., 43(6)*:1869-1878, 2025.

RESUMEN: La visualización efectiva de las estructuras del sistema nervioso central es fundamental para la enseñanza de la neuroanatomía, especialmente a nivel de pregrado, donde el acceso a especímenes frescos y tecnologías avanzadas de imagen suele ser limitado. Las técnicas de tinción clásicas, como las desarrolladas por Sincke, Mulligan y Braak, han contribuido significativamente a la docencia anatómica; sin embargo, muchos de estos protocolos implican procedimientos complejos, el uso de químicos peligrosos o tiempos prolongados de fijación y procesamiento que dificultan su aplicación generalizada en contextos educativos. En respuesta a estas limitaciones, el presente estudio tuvo como objetivo desarrollar y evaluar una adaptación simplificada y de baja toxicidad de la técnica de Mailland para teñir tejido cerebral fijado en formalina. Se utilizaron dos cerebros bovinos (*Bos taurus*), seccionados en cortes parasagitales de 3 mm de espesor, obteniéndose un total de 18 láminas, que fueron teñidas con cloruro férrico y ferrocianuro potásico en concentraciones variables (1 %, 0,75 % y 0,5 %) y diferentes tiempos de inmersión. La pigmentación azul resultante permitió una clara diferenciación entre la sustancia gris y la blanca, observándose una mayor intensidad de tinción a medida que aumentaban la concentración y el tiempo de exposición. El Grupo 1 (1 % durante 5 min y 1 min, respectivamente) mostró la coloración más intensa, mientras que el Grupo 6 (0,5 % durante 1 min y 30 s) aún presentó uncontraste confiable, destacando la eficacia del método incluso en condiciones mínimas. El protocolo adaptado no requiere equipamiento especializado, se realiza a temperatura ambiente y emplea reactivos de fácil acceso, lo que lo hace altamente aplicable en laboratorios académicos con infraestructura limitada. Esta técnica representa, por tanto, una valiosa herramienta pedagógica para mejorar la enseñanza de la neuroanatomía mediante una mejor visualización estructural.

PALABRAS CLAVE: Tinción cerebral; Técnica de Mailland; Neuroanatomía; Educación anatómica.

REFERENCES

Alston, R. L. A batch staining method for brain slices allowing volumen measurements of grey and white matter using an image analyzing computer (Quantimet 720). *Stain Technol., 56(4)*:207-13, 1981.

Azu, O. O.; Naidu, E. C. S. & Haghegh, E. Y. Morphological relationship between the superficial cortical and deep grey matter structures in adult human brains: A cadaveric study. *Int. J. Morphol., 37(4)*:1437-43, 2019.

Barnard, J. W.; Roberts, J. O. & Brown, J. G. A simple macroscopic staining and mounting procedure for wet sections from cadaver brains. *Anat. Rec., 105(1)*:11-7, 1949.

Blair, D. M.; Davies, F. & McClelland, E. W. On the nature of certain macroscopic staining reactions of the brain. *J. Anat., 66(Pt. 4)*:478-85, 1932.

Braack, H. Simple and durable staining of thick sections of the human brain for macroscopic study. *Stain. Technol., 53(2)*:87-9, 1978.

Ccorahua-Rios, M. S.; Chanco-Tupa, E.; Tacar-Huamani, J. L. & Santander-Cahuantico, A. C. Coloración encefálica como método de elaboración de material didáctico en neuroanatomía. *Rev. Peru. Morfol., 1(1)*:11-2, 2020.

Forlizzi, V.; Miranda-Solís, F.; Pérez Cruz, J. C.; Ccahuantico-Choquevilca, L. A.; Morán, G. & Baldoncini, M. Tinción de Mulligan en neuroanatomía: protocolización de la técnica. *Rev. Argent. Anat. Online, 11(2)*:31-4, 2020.

LeMasurier, H. E. Simple method of staining macroscopic brain sections. *Arch. Neurol. Psychiatr., 34(5)*:1065-7, 1935.

Loftspring, M. C.; Smanik, J.; Gardner, C. & Pixley, S. K. Selective gray matter staining of human brain slices: Optimized use of cadaver materials. *Biotech. Histochem., 83(3-4)*:173-7, 2008.

Mainland, D. Uber makroskopiche faerburg von Gehirnprraeparaten mit Berlinblau. *Anat. Anz., 65*:85-8, 1926.

Meneses, M. S.; Montano Pedroso, J. C.; Fuzza, R. F. & Milano, J. B. Análise comparativa de cortes de encéfalos humanos com coloração por três técnicas diferentes. *Arqu. Neuro-Psiquiatr., 62(2a)*:276-81, 2004.

Merini, T. T.; Krum, L. K.; Colman, J.; Bach, R. W.; Blood, M. R. Y. & Camargo, C. H. F. Comparação entre duas técnicas de coloração de encéfalos humanos. *Publ. UEPG Cienc. Biol. Saúde, 20(2)*:99-104, 2014.

Mulligan, J. H. A method of staining the brain for macroscopic study. *J. Anat., 65(Pt. 4)*:468-472.1, 1931.

Roberts, M. & Hanaway, J. Preparation of brain slices for macroscopic study by the copper sulphate-phenol-ferrocyanide technique. *StainTechnol. 44(3)*:143-6, 1969.

Rodrigues, H. Técnicas Anatômicas. 4th ed. Vitória, GM Gráfica e Editora, 2010.

Skopnik-Chicago, M.; Poblete-Cordero, K.; Zamora, N.; Bastías, R. & Lizana, P. A. Comparison of haptic and biometric properties, bacterial load, and student perception of fixative solutions: formaldehyde versus chilean conservative fixative solution with and without formaldehyde in pig kidneys. *Anat. Sci. Educ.,14(6)*:836-46, 2021.

Suriyaprapadilok, L. & Withyachumnarnkul, B. Plastination of stained sections of the human brain: comparison between different staining methods. *J. Int. Soc. Plastination, 12(1)*:27-32, 1997.

Villegas-Gomez, G. A.; Figueredo, L. F.; Ramirez, A. D.; Quiroga-Padilla, P. J. & Rueda-Esteban, R. Macroscopic brain gray matter staining: historical protocol overview and neuroanatomy learning applications in second-year medical students. *Front. Neuroanat., 17*:1227933, 2023.

Wu, M. & Kiernan, J. A. A new method for surface staining large slices offixed brain using a copper phthalocyanine dye. *Biotech. Histochem., 76(5-6)*:253-5, 2001.

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