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# Advances in thin tissue Golgi-Cox impregnation: Fast, reliable methods for multi-assay analyses in rodent and non-human primate brain

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## HIGHLIGHTS

- We describe thin tissue 'Golgi-Cox' methods that allow fast impregnation without significant crystallization artifact.
- These methods are compatible with the tissue fixatives acrolein, glutaraldehyde, or paraformaldehyde.
- Fixative perfusion allows using alternate sections for analyses such as immunohistochemistry and electron microscopy.
- We also describe immunohistochemistry and Golgi impregnation in the same tissue section without de-impregnation protocols.
- We detail advantages and disadvantages to guide for the investigator interested in tailoring Golgi-Cox for their own use.

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## ABSTRACT

In 1873 Camillo Golgi discovered a staining technique that allowed for the visualization of whole neurons within the brain, initially termed 'the black reaction' and is now known as Golgi impregnation. Despite the capricious nature of this method, Golgi impregnation remains a widely used method for whole neuron visualization and analysis of dendritic arborization and spine quantification. We describe a series of reliable, modified 'Golgi-Cox' impregnation methods that complement some existing methods and have several advantages over traditional whole brain 'Golgi' impregnation. First, these methods utilize 60–100 μm thick brain sections, which allows for fast, reliable impregnation of neurons in rats (7–14 days) and non-human primates (NHP) (30 days) while avoiding the pitfalls of other 'rapid Golgi' techniques traditionally employed with thin sections. Second, these methods employ several common tissue fixatives, resulting in high quality neuron impregnation in brain sections from acrolein, glutaraldehyde, and paraformaldehyde perfused rats, and in glutaraldehyde perfused NHP brain tissue. Third, because thin sections are obtained on a vibratome prior to processing, alternate sections of brain tissue can be used for additional analyses such as immunohistochemistry or electron microscopy. This later advantage allows for comparison of, for example, dendrite morphology in sections adjacent to pertinent histochemical markers or ultrastructural components. Finally, we describe a method for simultaneous light microscopic visualization of both tyrosine hydroxylase immunohistochemistry and Golgi impregnation in the same tissue section. Thus, the methods described here allow for fast, high quality Golgi impregnation and conserve experimental subjects by allowing multiple analyses within an individual animal.



*Abbreviations:* 3-D, 3-dimensional; 6-OHDA, 6-hydroxydopamine; ABC, avidin biotin peroxidase complex; BrdU, bromodeoxyuridine; DA, dopamine; D1R, dopamine D1 receptor; D2R, dopamine D2 receptor; DAB, 3,3′-diaminobenzidine tetrahydrochloride; DMSO, dimethyl sulfoxide; EM, electron microscopy; IHC, immunohistochemistry; immunoEM, immunoelectron microscopy; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MSN, medium spiny neuron; NGS, normal goat serum; NHP, non-human primate; PB, phosphate buffer; PF, paraformaldehyde; PSD-95, post-synaptic density-95 kDa protein; RT, room temperature; SG, vector slate grey; TH, tyrosine hydroxylase; Tx, Triton X-100.

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## 1. Introduction

In 1873, Camillo Golgi discovered the basic method of staining neurons in the nervous system that was initially termed 'the black reaction' (*la reazione nera*) and is now referred to as Golgi staining. After laborious attempts to stain elements of nervous tissue, Golgi uncovered a process involving nervous tissue hardening in potassium dichromate and impregnation with silver nitrate (Golgi, 1873). The original method required immersion of tissue in the potassium dichromate for several months and subsequent impregnation in silver nitrate for several additional days. One of the main strengths of the Golgi method is that it is capricious in that it stains only approximately 1–10% of neurons in any one select region (Shankaranarayana et al., 2004). This capricious staining allows a panoramic visualization of virtually all parts of an individual neuron including the soma, axon, dendrites, and dendritic spines.

Understanding brain connectivity, function and structural modifications associated with various pathological conditions has been of interest for more than a century. While neuroscientists have developed a vast array of methods to probe neuronal architecture (for review see Lanciego and Wouterlood, 2011), Golgi impregnation remains a frequently used method for whole neuron visualization and elegant detailed analysis of dendritic arborization and dendritic spine phenotypes (e.g.: Robinson and Kolb, 1999; Diana et al., 2006; Marchetti et al., 2009; Srivastava et al., 2009; Hamilton et al., 2010; Li et al., 2012; Pinto et al., 2012; Krugers et al., 2012). In recent decades, there has been interest from neuroscientists to apply this classical approach of staining whole neurons in conjunction with newer approaches (Somogyi et al., 1981; Anderson and Felten, 1982; Buller and Rossi, 1993; Diana et al., 2006; Pilati et al., 2008; Spiga et al., 2011; Pinto et al., 2012) and, thus, numerous variations of Golgi impregnation have been developed. Each of the variant approaches has advantages and disadvantages compared to the classic Golgi approach. One specific disadvantage commonly associated with thin tissue impregnation has been the crystallization artifacts that are endemic in the 'rapid Golgi' method of staining (Gabbott and Somogyi, 1984; Friedland et al., 2006). These artifacts are thought to be due to a nonselective reaction between potassium dichromate and silver nitrate, resulting in the formation of bulk crystals on the surface of the specimen (Pasternak and Woolsey, 1975).

In the experiments described in this paper, we use a variation of the original Golgi recipe, termed Golgi-Cox (e.g.: Cox, 1891; Van der Loos, 1956; Glaser and Van der Loos, 1981), in which silver nitrate is replaced with mercury chloride to foster the impregnation of neurons. We describe a variation of the Golgi-Cox method, similar to that detailed by Landas and Phillips (1982), which reliably and effectively impregnates neurons in thin brain tissue sections (100 μm) without significant crystallization artifact. We detail here the extensive characterization of this modified Golgi-Cox method that provides high quality, reliable Golgi impregnation of neurons in thin brain tissue sections from either rat or non-human primate (NHP). We have characterized the advantages and caveats of the Golgi-Cox method associated with various fixatives including paraformaldehyde (PF), acrolein, and glutaraldehyde. Fixative perfused sections are also compared with saline perfused sections, which were Golgi impregnated as thin tissue sections (100 μm) or as blocks (~7 mm). Further, because thin sections were obtained on a vibratome prior to Golgi-Cox processing, we detail the reliability and potential caveats of using alternate sections of brain tissue for additional analyses including immunohistochemistry (IHC) and electron microscopy (EM). Finally, we describe a method for performing and visualizing, with light microscopy, immunohistochemically stained and Golgi impregnated neurons in the same tissue section. This approach complements and extends the utility of dual Golgi/IHC reported previously by Buller and Rossi (1993), Spiga et al. (2011), and Pinto et al. (2012) using different experimental conditions.

Overall, the advantage of employing Golgi impregnation with thin tissue sections provides value by (1) eliminating the need for impregnating the entire brain, thus allowing for a more expeditious staining of neurons and (2) conserving the number of animals required for a study by allowing for the use of the alternate brain sections, taken in series, for Golgi impregnation and additional analyses such as IHC or EM. The ability to obtain multiple measurements from each animal in a single experiment offers advantages including a determination of the strength of the association between the dependent measures, determining which of the dependent measures is most important, and an examination of the effects of the covariates. Throughout the manuscript we highlight several technical details associated with Golgi-Cox impregnation so that any investigator may tailor their various assays to work in tandem.

## 2. Materials and methods

### 2.1. Animals

Adult male Sprague Dawley rats (Harlan Laboratories, Indianapolis, IN), weighing 225–250 g at the start of the experiment, were housed in groups of two. Food and water were available ad libitum in their home cages. Rats were maintained on a 12 h light/dark cycle with lights on at 07:00 h. Rhesus monkeys (*Macaca mulatta*), weighing 6–20 kg at the start of the experiment, were housed separately in home cages and exposed to a 12 h light/dark cycle. They were fed daily in amounts appropriate for the size and age of the animals and water was available ad libitum. All studies were carried out in accordance with the Declaration of Helsinki and with the Institute for Laboratory Animal Research of the National Academy of Science *Guide for the Care and Use of Laboratory Animals* (8th edition, revised 2011) and were approved by the Michigan State University, University of Cincinnati, and University of California San Francisco Institutional Animal Care and Use Committees. All efforts were made to minimize the number of animals used and their pain or discomfort.

### 2.2. Preparation of the Golgi-Cox fixative solution

For all cases described herein, the Golgi-Cox fixative solution was prepared such that the final concentration of mercury chloride ($HgCl_2$, Fisher Scientific, Pittsburgh, PA), potassium chromate ($K_2CrO_4$, Sigma–Aldrich Corp., St. Louis, MO), potassium dichromate ($K_2Cr_2O_7$, Sigma–Aldrich Corp., St. Louis, MO) was 1%; each was dissolved in distilled water. For example, to prepare a 1 L solution of Golgi-Cox fixative solution, 10 g of mercury chloride was first dissolved into 200 ml of distilled water at 80 °C. The solution was allowed to cool to room temperature (RT) and was then slowly added to an equal volume of a 5% solution of potassium dichromate (e.g.: 10 g of potassium dichromate was dissolved into 200 ml of distilled water at RT). To this a 1.67% solution of potassium chromate (e.g.: 10 g dissolved into 600 ml distilled water at RT) was slowly added while stirring. The solution was wrapped in aluminum foil to protect it from light and allowed to sit for 12 h at RT. Care was used when using aluminum foil as a light barrier with Golgi-Cox methods as aluminum is highly soluble in this fixative and shreds of dissolved foil that could fall into the sample containers will inhibit Golgi impregnation. The solution was decanted into sample containers,