Published in final edited form as:

J Neurosci Methods. 2009 January 30; 176(2): 72–77. doi:10.1016/j.jneumeth.2008.08.021.

A new use for long-term frozen brain tissue: Golgi impregnation

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Abstract

The study of dendritic spine shape and number has become a standard in the analysis of synaptic transmission anomalies since a considerable number of neuropsychiatric and neurological diseases have their foundation in alterations in these structures. One of the best ways to study possible alterations of dendritic spines is the use of Golgi impregnation. Although usually the Golgi method implies the use of fresh or fixed tissue, here we report the use of Golgi-Cox for the staining of human and animal brain tissue kept frozen for long periods of time. We successfully applied the Golgi-Cox method to human brain tissue stored for up to 15 years in a freezer. The technique produced reliable and reproducible impregnation of dendrites and dendritic spines in different cortical areas. We also applied the same technique to rat brain frozen for up to one year, obtaining the same satisfactory results. The fact that Golgi-Cox can be successfully applied to this type of tissue adds a new value for hundreds of frozen human or animal brains kept in the freezers of the laboratories, that otherwise would not be useful for anything else. Researchers other than neuroanatomists, i.e. in fields such as biochemistry and molecular biology can also benefit from a simple and reliable technique that can be applied to tissue left from their primary experiments.

Keywords

Cortex; Dendritic spine; Neuroanatomy; Neuropathology; Striatum; Schizophrenia; Synapse

INTRODUCTION

In spite of being developed more than 100 years ago by Camillo Golgi, and popularized by Santiago Ramon y Cajal, the Golgi method is still a very useful and widely used technique that reveals the fine structure of neurons, glia and their processes, both in the normal and pathological nervous system. The descriptions of neurons as we know them today were possible thanks to the use of this impregnation technique developed more than 100 years ago. One of the fine structures that were first revealed by the use of the Golgi method and that caught the eye of Santiago Ramon y Cajal were the dendritic spines. They were first described by Cajal as small thorns that projected from the dendrites of cerebellar Purkinje cells (Ramon y Cajal, 1888). Although thought to be artifacts in the beginning, dendritic spines are today known to be real centers of information processing and are able to control their own protein synthesis and degradation (see as a review Halpain et al., 2005). Dendritic spines are plastic structures

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that receive the majority of excitatory synapses. They actively change in number, shape, and size in response to subtle variations in their extracellular milieu due to a number of different factors (synaptic activity, changes in the composition of the extracellular space, use of drugs (prescription and non-prescription), and disease, see Fiala et al., 2002 as a review), thus altering neuronal activity. Of particular interest for us are the last two factors.

Since the introduction of neuroleptic drugs for the treatment of schizophrenia back in the 1950s, a number of studies have pointed out the effect of these medications on dendritic spines (Kelley et al., 1997). It has been reported that dendritic spines are altered even before apparent damage in the parent neurons in neurodegenerative diseases such as Parkinson's, Huntington's or Alzheimer's disease (Fiala et al., 2002; Spires et al., 2004; Gerfen, 2006; Baloyannis et al., 2007), and also in diseases in which such "macroscopic" alterations of neurons do not occur or are much subtler, such as schizophrenia and depression (Glantz and Lewis, 2000; Hill et al., 2006). It is important to determine what kind of alterations occur in dendritic spines (i.e. number, size, shape, or abnormal origin within the neuron) in diseases like schizophrenia, where alterations in the synaptic transmission of several neurotransmitters as well as reductions in the number or size of dendritic spines have been reported (Kelley et al., 1997; Glantz and Lewis, 2000). Moreover, it is important to determine if those alterations are due to the disease itself or are caused by the use of antipsychotic drugs. This is why the use and analysis of human brain tissue from patients that were never exposed to antipsychotics, or from patients that were off-medication for a period of time before the harvest of the tissue becomes essential. Brain tissue from untreated subjects with schizophrenia is rare and is usually kept in a freezer for very long periods of time. Fresh frozen tissue has many applications in molecular biology and biochemistry experiments (in situ hybridization, western-blot, DNA or RNA extraction, etc) but has little application in neuroanatomy studies because of the lack of good morphology due to the preservation conditions. Many brain banks or collections accumulate and store hundreds of frozen brains or dissected brain areas for very long periods of time with no apparent use for them in morphological studies, and in a similar manner animal brains are kept in freezers after being subjected to different experimental approaches such as the evaluation of the administration of drugs on the expression of a particular protein or gene. Although the Golgi method has only been successfully used with fresh or fixed tissue, here we report a new use for this technique using frozen-stored tissue. We used one of the variations of the original Golgi recipe, named Golgi-Cox (Glaser and Van der Loos, 1981) and report its capability to produce reliable and reproducible impregnation of neurons and neuronal processes (dendrites and dendritic spines) in human brain samples kept frozen for different periods of time (1 year and 15 years). We also compare this impregnation in frozen tissue to that obtained in tissue kept in fixative for the same period of time. This technique is also applicable to frozen animal brains, which we also describe. Some of these results have been recently communicated in abstract form (Melendez-Ferro et al., 2006).

MATERIALS AND METHODS

Samples and technique

For our purpose we used control human brain samples from different cortical areas (Brodmann Areas 9, 10, and 11, which are known to be affected in schizophrenia) obtained from the Maryland Brain Collection (Maryland Psychiatric research Center, University of Maryland at Baltimore, Maryland, USA). These brain samples have been stored either frozen at -80°C (for comparative purposes we used samples frozen for up to 1 year and samples frozen for up to 15 years), or in fixative (10% neutral buffered formalin) for up to 15 years. Non-fixed rat brain tissue stored frozen for up to 1 year was also used. To perform the Golgi impregnation, a Golgi-Cox-based kit (FD Rapid GolgiStain™) was purchased from FDNurotechnologies, Inc. (Ellicott City, MD, USA). To our knowledge, this is the first time that the use of Golgi-Cox in

long-term frozen human or rat brain tissue has been reported. All procedures were in accordance with Institutional Review Board (IRB) and Institutional Animal Care and Use Committee (IACUC) approved protocols.

Pretreatments

Prior to impregnation, samples were subjected to different pretreatments depending on the storage conditions. Frozen samples from human and rat brains were thawed at room temperature in 0.1M phosphate buffer pH 7.4 (PB) and rinsed several times in PB prior to immersion in the chromation solution. Fixed tissue was subjected to either 1) no pretreatment, 2) a pretreatment consisting in the incubation of the tissue in citrate buffer pH 6.0 for two hours in an oven at 80°C, 3) a pretreatment with 1% sodium borohydride in PB for 2 hours, or 4) a combination of pretreatments 2 and 3 (1 hour each sequentially).

Impregnation procedure

After the samples were thawed or subjected to the different pretreatments, they were immersed in the chromation solution which consists of a mixture of mercury chloride + potassium dichromate + potassium chromate, following the instructions of the manufacturer. The chromation solution was replaced after the first 12 hours, and the samples were kept in the fresh solution in the dark for 2 or 3 weeks in the case of rat brain, or 3 weeks in the case of human brain. Brain samples were then transferred to a cryoprotectant solution and kept at 4°C for a minimum of 1 week, replacing the cryoprotectant solution after the first 48 hours. After that, the pieces of tissue were blocked and frozen using powdered dry ice, cut at 150 µm in thickness using a sliding microtome, and sections were mounted on gelatin-subbed slides and allowed to completely dry on a warm plate at 33–35°C, for 5 to 6 days.

For the final development, slides were rinsed two times in distilled water and placed in the developing solution (an ammonium hydroxide-based solution) for 10 minutes. After developing, slides were rinsed in distilled water, dehydrated in ascending series of ethanol, cleared in xylene, and coverslips were applied to the slides using Eukitt (Electron Microscopy Sciences, PA, USA).

Photography

Slides were observed on a fully motorized Nikon Eclipse 90i microscope (Nikon Instruments Inc., NY, USA), and photographed using a Photometrics Cool Snap HQ digital camera (Photometrics, AZ, USA). Stacks of microphotographs and 3D reconstructions were obtained and assembled using the NIS Elements AR Imaging Software v2.3 (Nikon Instruments Inc.).

RESULTS

In this study we describe for the first time the suitability of frozen human and rat brain tissue for its impregnation with Golgi-Cox, using a commercially available kit. For this purpose we qualitatively compared the impregnation obtained in human brain tissue frozen for 1 year with that obtained with the same type of tissue frozen for 15 years. We also show the results obtained in rat brain tissue frozen for 1 year and subjected to the same type of procedure. Finally, we describe the results obtained when long-term fixed human tissue is subjected to several pretreatments, and processed using the Golgi-Cox method.

Frozen rat brain tissue

The qualitative analysis of the impregnation of rat brain tissue that was frozen for 1 year prior to the chromation step shows an excellent impregnation in both cell bodies and processes, including dendritic spines. The quality of impregnation was very similar regardless of the

chromation time (2 or 3 weeks), so our description of the results obtained in the rat brain will be based on brains that were impregnated for 3 weeks (Fig. 1). Although the unspecific precipitation of some heavy metals could potentially occur, this does not constitute a major obstacle for the identification of the mentioned structures. Fig. 1A shows the cell body of a pyramidal cell in the rat cortex, as well as basilar and apical dendrites covered with spines. When high magnification is used, the different types of dendritic spines can be easily identified. Fig. 1B shows a micrograph of an apical dendrite and some of its secondary branches from a cortical neuron. Mushroom-shaped and thin spines are abundant. Fig. 1C shows a low magnification of the striatum with several impregnated neurons. No impregnated perineuronal astrocytes are observed that obscure the visualization of the neurons. In addition to mushroom and thin spines, branched spines can also be observed in a detail from the striatum (Fig. 1D).

Frozen human brain tissue

There were no differences in the quality of impregnation in the samples of human brain tissue analyzed regardless of the time in the freezer, or the region examined. Cell bodies as well as dendrites and dendritic spines were impregnated, and there was minimal background and few large deposits of heavy metals that could obscure these structures (see Figs. 2, 3). Three cortical brain regions (BA9, BA10, and BA11) and two time points (1 year and 15 years) are compared here. In an example of BA9 frozen for 1 year (Figs. 2A–D), pyramidal neurons with emerging dendrites covered with spines are observed (Fig. 2A). At higher magnification (Fig. 2B) mushroom, sessile and filopodia-type spines are mainly observed. The use of the NIS Elements AR Imaging software allows the three dimensional reconstruction of the two neurons (Fig. 2C) shown in Fig. 2A. The variety of dendritic spines in this region can be observed in Fig. 2D, where in addition to the same types of spines observed in Fig. 2B, branched spines can also be observed emerging from this dendrite (Fig. 2D). Similar types of dendritic spines are observed in BA10 frozen for 1 year (Figs. 2E, F). Fig. 2E (BA10 frozen for 1 year) shows an example of an impregnated neuron with emerging dendrites covered with spines. At higher magnification filopodia-type, thin and mushroom-shaped spines can be observed on this dendrite (Fig. 2F).

An example of a low magnification of a section of BA10 frozen for 15 years (Fig. 3A) shows the presence of numerous Golgi-impregnated neurons with impregnated dendrites. As it can be seen in Fig. 3A, some of these impregnated dendrites travel for a considerable distance from the parent neuron. Higher magnification of the same neuron reveals that the impregnated dendrites are profusely covered with dendritic spines (Fig. 3B). In a similar manner to BA10 frozen for 1 year, thin and filopodia-type are the predominant types of spines shown in this example of BA10 frozen for 15 years (Fig. 3C). The application of a software package reveals the real topography of dendrites that cannot be seen in a two dimensional image (Fig. 3D). However, thin and filopodia-type are not the only types of spines observed in BA10 frozen for 15 years. As it is shown in Fig. 3E, mushroom as well as branched spines can also be observed in this brain region. On the other hand, filopodia-type as well as branched spines are the main types of dendritic spines observed in this example of BA11 frozen for 15 years (Fig. 3F).

Long-term fixed human tissue and pretreatments

We decided to use the Golgi-Cox method on long-term fixed human tissue trying previously some pretreatments (citrate buffer and sodium borohydride) that are commonly used to unmask antigens in immunohistochemistry, to assess if they could have any effect in the impregnation of dendrites and dendritic spines. Tissue fixed for 15 years in formalin showed unsatisfactory impregnation and no improvement was observed even after being subjected to these different pretreatments. Fixed tissue not subjected to pretreatments simply showed the presence of some short “processes” with no obvious dendritic spines. Only some “thickenings” in these processes can be observed (Fig. 4A). The same pattern of impregnation was obtained in tissue that was

pretreated with citrate buffer (Fig. 4B). Finally, the use of sodium borohydride alone (Fig. 4C) or in combination with citrate buffer (Fig. 4D) merely reduced the cells to round dark-stained structures.

DISCUSSION

Here we report for the first time the validity of the use of an old impregnation technique for the study of human and animal brain tissue that has been stored frozen for long periods of time. Our results show that Golgi-Cox impregnation of frozen human or animal brain tissue is suitable for the analysis of certain parameters such as dendrite length and branching pattern, dendrite spine counts and the analysis of dendritic spine morphology. This analysis can be performed currently in a more reliable and accurate manner with the use of motorized microscopes that allow the performance of stacks of micrographs in the Z axis (basic for the three dimensional reconstruction of neurons) in combination with software applications for the quantification of such parameters in the resulting micrographs (see i.e. Garcia-Lopez et al., 2006).

After the qualitative analysis of our impregnated frozen human material, the length of time in the freezer does not seem to affect the impregnation of neurons and processes. This is illustrated by the observation that different types of spines appear in a particular brain region regardless of the time of permanence in the freezer, and for the fact that the same types of dendritic spines appear in different brain regions stored for the same period of time (see Fig. 2), or in the same brain region stored for different periods of time (compare Figs. 2E, F and 3A–E). Altogether these prove that the impregnation is specific and not due to artifacts. Golgi-Cox is the chromation technique of choice when fresh or slightly fixed tissue is available (Glaser and Van der Loos, 1981; Buell, 1982; Rosoklija et al., 2003), but here we show that it can be applied when the chromation solution is not available at the same time as the tissue. As illustrated in Figs. 2 and 3, our impregnated material is also devoid of any precipitate that could possibly obscure the identification of the dendritic spines. Additionally, the results obtained with the frozen rat brain show that animal brain tissue is also suitable for this kind of procedure. Animal brains are often kept in the freezer (many times after having been treated experimentally), and very useful data on dendritic morphology and dendritic spines can now be obtained after the analysis of brains impregnated with Golgi-Cox.

Many different variations of the original Golgi recipe have been developed over the years (Cox, 1891; Kopsch, 1896; Ramon y Cajal and De Castro, 1933; Colonnier, 1964), and many authors have reported very different results using the same variation. In our case, as in the case of other researchers (Zhang et al., 2003) the use of Golgi-Cox in human tissue that was fixed in formalin for years revealed to be totally unsatisfactory, even after the use of unmasking agents to remove the excess of fixation produced over the years. Other types of Golgi impregnation such as Rapid Golgi or Golgi-Kopsch have proven to give satisfactory results when applied to tissue that was fixed for different periods of time in different fixative solutions (Buell, 1982; Rosoklija et al., 2003).

The Golgi method has many inherent peculiarities such as non-uniform, incomplete and/or random impregnation of neurons and/or dendrites and dendritic spines. Moreover different types of Golgi methods need to be used depending on the conditions of the tissue (see i.e. Rosoklija et al., 2003). That said it is clear that this technique is still a very useful tool for the study of the normal anatomy as well as the pathology of the central nervous system, and can provide us with an estimate of the functional status of a particular nucleus or brain region. The utility of this old technique and of the new application that we are proposing is even more evident with the development of computer software and hardware that allow a more thorough

analysis of these structures (see i.e. Garcia-Lopez et al., 2006; Perez-Costas et al., 2007 and Figs. 2C and 3D).

The success obtained with this technique in frozen tissue will allow the use of long-term stored brain samples from brain banks and collections for the study of neurological and neuropathological conditions such as schizophrenia. As an example, in the case of schizophrenia, the ability to use frozen-stored tissue from patients that were not exposed to antipsychotic drugs will allow the study of the “dynamics” of the dendritic spines without the confounding effects of the medication. On the other hand, those researchers who do not have neuroanatomy as their primary field of research, such as researchers in the fields of biochemistry or molecular biology, can benefit from a simple (the procedure is simple and needs minimal infrastructure) but at the same time reliable (produces good results) methodology that can be applied to brain tissue (human or animal) left from their primary experiments.

Acknowledgements

The authors wish to thank the Maryland Brain Collection (Maryland Psychiatric Research Center, University of Maryland at Baltimore, Baltimore, MD) for providing us with the tissue used in this study. This work was supported by NIH grants MH66123 and MH060744 to RCR.

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Fig. 1.

Golgi-Cox impregnation in the cortex (A, B) and striatum (C, D) of a rat brain kept for 3 weeks in the chromation solution. **A)** Pyramidal neuron of the cortex of the rat showing the classical cell body morphology as well as several basilar dendrites covered with dendritic spines (thin arrows). **B)** Detail of the apical dendrite from the same neuron in A showing secondary branches profusely covered with dendritic spines. **C)** Low magnification of the striatum of the rat showing Golgi-impregnated medium spiny neurons. It is possible to appreciate the dendritic spines even at this magnification (thin arrows). **D)** High magnification of the box in C, where the dendritic spines can be observed with much more detail and their morphologies are easily distinguished. Outlined white arrowheads: mushroom spines; outlined black arrows: thin spines; empty white arrowheads: branched spines. Magnification: 20× in A, C; 60× in B, D.

Fig. 2.

Golgi-Cox impregnation in BA9 (A-D) and BA10 (E, F) of human cortex that was kept in a -80°C freezer for 1 year. **A)** Low magnification of two neurons (outlined white arrows) in BA9 showing impregnated dendrites covered with dendritic spines (thin arrows). **B)** Detail of the box in A showing the different morphologies of the dendritic spines. **C)** Three dimensional reconstruction of the two neurons shown in A (outlined white arrows). **D)** Detail of a different dendrite from another neuron in BA9 showing the variety of shapes of the dendritic spines. **E)** Golgi-impregnated neuron in BA10 showing stained dendrites with dendritic spines (box). **F)** Detail of the fragment of the dendrite boxed in E showing the definition of the Golgi impregnation and the diversity of morphologies of the spines. Outlined white arrowheads: mushroom spines; outlined black arrow: thin spine; empty white arrowheads: branched spines; empty white arrows: filopodia; empty black arrows: sessile spines. Magnification: $20\times$ in A, E; $60\times$ in D, F; $100\times$ in B.

Fig. 3. Golgi-Cox impregnation in BA10 (A-E) and BA11 (F) of human cortex that was kept in a -80°C freezer for 15 years. **A)** Low magnification micrograph in BA10 showing impregnated neurons, some of which have very long impregnated processes (thin arrows). **B)** High magnification of the area boxed in A showing a dendrite covered with dendritic spines (thin arrows). **C)** Detail of the boxed fragment of dendrite in B showing the different types of dendritic spines in this segment. **D)** Three dimensional reconstruction of the same fragment of dendrite than in C, showing how tortuous this dendrite is. **E)** Detail of a different Golgi-impregnated fragment of dendrite showing the variety of shapes of the dendritic spines. **F)** High magnification of a Golgi-impregnated dendrite in human BA11 showing the fine detail of the dendritic spines in this fragment of dendrite. Note that the quality of impregnation obtained in these samples is very similar to that observed in the samples that were processed for Golgi-Cox after only 1 year in the freezer. The halo-like appearance in some figures (C, F) is due to the multiple stacks of micrographs required to compose the image, due to the tortuous shape of the dendrites. Outlined black arrows: thin spines; empty white arrowheads: branched spines; empty white arrows: filopodia. Magnification: $10\times$ in A; $20\times$ in B; $60\times$ in C, E, F.

Fig. 4.

Golgi-Cox impregnation of human cortex stored for 15 years in formalin and subjected to different pretreatments before the chromation step (see materials and methods). **A)** BA11 with no pretreatments. **B)** BA10 after citrate buffer pretreatment. **C)** BA9 after sodium borohydride pretreatment. **D)** BA11 after a combination of citrate buffer and sodium borohydride. No recognizable structures are observed in the tissue stored in this condition even after the application of different pretreatments. Magnification is 10 $\times$ in all figures.