Quick Golgi method: Modified for high clarity and better neuronal anatomy

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The Golgi methods have long been used to study the neuronal soma, axons, dendritic arborization and spines. The major concerns of the Golgi method have been its unpredictable nature (inconsistency of impregnation of the stain), time consumed, tissue hardening and clear background, resulting in several modifications to improve the cellular visualization. In the present work we describe a modification of the rapid-Golgi method that takes the benefit of perfusion fixation (with rapid-Golgi solution) then post-fixation in the same fixative for 36 h followed by 36 h impregnation in aqueous AgNO₃ followed by vibratomy. This modification is simpler, faster and inexpensive, provides a consistent staining of neurons with good resolution of neuronal soma, dendritic arborization as well as spines with much reduced formation of silver chromate crystals and background in just 3 days.

Keywords: Cerebellum, Cerebral cortex, Dendritic spines, Purkinje neurons, Pyramidal neurons, Rapid-Golgi fixative

Camillo Golgi in 1873 discovered that impregnation of brain slices with silver chromate solution resulted in staining of a small population of neurons in their entirety¹. This is known as Golgi method and continues to be extremely useful and very frequently used even today for studying the neuronal architecture. Over the years the method has been variously modified. The classical Golgi method was modified by Cajal referred as rapid Golgi staining method². Cajal extensively used this method and demonstrated the previously unimagined neuronal morphology throughout the nervous system, most of which continues to be accepted. However, Golgi method’s importance was best felt when Golgi’s so called artefacts with no physiological relevant structures on the dendritic surfaces turned out to be the dendritic spines in the studies of Cajal. He identified the real value of Golgi method and was first to describe spines as small thorns that protruded from the dendrites of cerebellar Purkinje neurons²,³. Now the dendritic spines are known as actual centres of information processing with the ability to regulate their own protein synthesis and degradation⁴,⁵. Dendritic spines are proposed as the primary sites of synaptic plasticity⁶, their number, shape and size changes in response to variations in their extracellular environment⁷, environmental changes⁸-¹⁰ and with evolution¹¹,¹². Most researchers today rely on a two-step procedure of Golgi staining, chromation (potassium chromate and/or potassium dichromate solution) and silver nitrate impregnation which subsequently allow the formation of silver chromate crystals visualized as brownish-black structures¹³. The only concern of the Golgi method has been its unpredictable nature. This has resulted in several modifications in terms of variation in solution composition and pH¹⁴-²¹, use of single sections for staining²²,²³, use of microwaves¹³,²⁴-²⁶, changing embedding media²⁷,²⁸, use of vibratome²⁹, coating of brain blocks with egg yolk¹³, application of vacuum³⁰, auto metallographic (AMG) enhancement³¹ and variation in temperature of the tissue incubating media²¹,²⁴-²⁶,³². Most modifications aimed at decreasing the time required for the procedure, reduced precipitation leading to a clearer background and uniform impregnation and uptake of the Golgi stain in the nervous tissue. Golgi staining method is useful for labelling cell profiles in the central nervous system, but takes several weeks. The long period of fixation turns the tissue brittle creating problems in sectioning. The deeply stained blood vessels appearing as background interfere in deciphering the neuronal structures. The present communication reports a simpler, faster and efficient modification of rapid-
Golgi staining involving the perfusion of animal with rapid Golgi fixative and post-fixation in the same fixative leading to a clear background, reduced blood vessels staining and clear spines and quicker than the earlier modifications.

Materials and Methods

Wistar rats (250-300 g) used in this study were raised in the Animal House of School of Studies in Neuroscience. The animals were housed in chambers at 25±2 °C room temperature, approximately 50% RH and 12:12 h L:D conditions. Pelleted standard rat feed and water was provided ad libitum. The experimental protocols were pre-approved by the Institutional Animal Ethics Committee (501/01/a/CPCSEA). A total of 27 male rats were used in this study.

Rapid-Golgi staining method

Rapid-Golgi fixative solution (RGF) was prepared as per Patro et al., viz., 5 g potassium dichromate (Himedia Laboratories, India), 5 g chloral hydrate (MERCK, India), 8 mL glutaraldehyde (CDH, India), 6 mL formaldehyde (Qualigens, India), 10 drops of dimethyl–sulphoxide (Qualigens, India) were mixed and final volume was made up to 100 mL with distilled water. Three rats were sacrificed by cervical dislocation followed by decapitation, their brains were dissected out and the cerebral cortex and cerebellum were removed. Approximately 1 cm³ tissue blocks both from cerebral cortex and cerebellar cortex (through vermis region) were directly immersed in Golgi fixative and kept in dark amber bottles, undisturbed, for 2 days followed by a second change of Golgi fixative for next 2 days. On the 5th day tissue blocks were rinsed briefly (2-3 times) with 0.75% aqueous solution of silver nitrate (Qualigens, India) and kept submerged in the same solution for 2 days in dark. The tissue blocks were again rinsed with 0.75% silver nitrate solution (2-3 times) and kept in the same solution for 3 days in dark. Subsequently, tissue pieces were washed properly and carefully in 70% alcohol to remove any precipitates and 100 µm thick sections were cut using a Leica Automatic Vibratome (VT1000S Leica Microsystems, Wetzlar, Germany). The slides were visualized using Leica DM6000 microscope equipped with Leica DFC 310 FX digital camera (Leica Microsystems, Wetzlar, Germany). The slides were visualized with Leica DM6000 microscope fitted with a digital camera DFC 310 FX (Leica Microsystems, Wetzlar, Germany).

Quick Golgi staining method (present modifications)

To validate the benefits of the proposed modifications in the rapid Golgi method, 12 male Wistar rats were anaesthetized with ether (Qualigens, India) vapors and brains were perfusion-fixed (transcardially) with 200 mL of phosphate buffer saline (PBS) to washout the blood followed by 100 mL of rapid Golgi fixative (P-RGF group) solution at the rate of 20 mL/min. Another group of 12 rats were perfused only with phosphate buffer saline (P-PBS group). After perfusion with PBS alone or PBS followed by rapid-Golgi fixative solution (P-RGF) the brains were dissected out and the cerebral cortex and cerebellum were removed. Approximately 1 cm³ tissue blocks from both P-PBS and P-RGF group rats were post-fixed in RGF and impregnated with 0.75% aqueous AgNO₃ solution in the following combinations (n=3): 12 h post-fixation followed by 24 or 36 h AgNO₃ impregnation; 24 h post-fixation followed by 24 or 36 h AgNO₃ impregnation; 36 h post-fixation followed by 12, 24 or 36 h AgNO₃ impregnation; 48 h post-fixation followed by 48 h AgNO₃ impregnation. On completion of the respective duration in AgNO₃ solution, tissues were washed properly in 70% alcohol to remove any precipitates and 100 µm thick sections were cut using a Leica Automatic Vibratome (VT1000S Leica Microsystems, Wetzlar, Germany). The slides were visualized using Leica DM6000 microscope equipped with Leica DFC 310 FX digital camera (Leica Microsystems, Wetzlar, Germany) and the LAS V4.2 multifocus module that automatically captured a stack of images at different focus positions across the depth of the neurons. These images were then combined into a single image all in-focus neuronal processes of the stack. The spine density of 220 neurons (110 Purkinje and 110 pyramidal neurons) was analyzed, in both the staining methods, using an ocular micrometer scale fitted in the eye piece of a microscope. For each neuron, 3 measurements were made. The data were analysed by t-test using SigmaStat version 3.5 for Windows. Values of P≤0.05 were considered significant.

Results

To get better results faster than the earlier modified Golgi methods, perfusion fixation with the rapid Golgi fixative solution followed by combinations of various durations of post-fixation and development with AgNO₃ have been tried in the present study. We then looked for low background, blood vessels
staining and crystallization with better cellular details like cell processes, spines, etc. (Table 1). Combination of 12/24 h and 24/36 h post fixation with rapid-Golgi fixative solution followed by AgNO₃ incubation could not develop much of the details. In P-PBS group no details could be seen (Fig. 1A and C, respectively), while in P-RGF group, the interneurons and Bergmann glial fibers were visible with moderate impregnation to the central part of the tissue blocks (Fig. 1B and D, respectively). The combination of 36 h post-fixation following perfusion with rapid-Golgi fixative solution and 36 h development with AgNO₃ produced wonderful visualization of cellular details. This combination resulted in minimum background, least appearance of Golgi stained blood vessels, complete cellular details (cell soma, axon, dendrites and spines) of Purkinje neurons and visibility of interneuron connections with main projection neurons (stellate, basket and Golgi cells with Purkinje neurons, Table 1; Fig. 1F) when compared with corresponding P-PBS group (Table 1; Fig. 1E). Post fixation for 48 h followed by 48 h development with AgNO₃ also produced similar results with hardly any further improvement in P-RGF group (Fig. 1H). However, perfusion with PBS alone did not provide any such details (Fig. 1G). As compared to 9 days in rapid Golgi staining procedure, this modification helps getting better results in 3 days. It is thus proposed that a combination of perfusion fixation with rapid-Golgi solution followed by 36 h post-fixation and 36 h AgNO₃ may be used, thus by the end of the 3rd day the tissue is ready for sectioning and visualization. The results thus obtained are better than the rapid-Golgi method with earlier modification that takes 9 days.

Further, the rapid-Golgi stained brain sections were compared with the present Quick Golgi stained brain sections of 36/36 h combination. Tissue sections of the present Quick Golgi method presented clearer details and contrasting cell profiles (Fig. 2B, D and F; 3B, D and F) as compared to the rapid-Golgi method (Fig. 2A, C and E; 3A, C and E). This difference in visibility of cell profiles among two different methods was due to reduced (or even no) staining of the blood vessels in the proposed Quick Golgi method resulting in a clear background thus enabling study of the complete cellular details. The rapid-Golgi stained blood vessels, prominently visible in the brain sections of animal perfused with PBS only ruled out the doubt that blood and its constituents are responsible for the background. Good camera lucida tracings were possible with prominent dendritic spines against a clear background in the proposed Quick Golgi stained sections (Fig. 2H and 3H). In

<table>
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<tr>
<th>Combinations</th>
<th>Impregnation to central part</th>
<th>Interneurons</th>
<th>Bergmann glial fibers</th>
<th>Purkinje cells/spines</th>
<th>Pyramidal cells/spines</th>
<th>Background</th>
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<tr>
<td>12 h fixative &amp; 24 h AgNO₃</td>
<td>P-RGF</td>
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<td>12 h fixative &amp; 36 h AgNO₃</td>
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<td>24 h fixative &amp; 24 h AgNO₃</td>
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<td>24 h fixative &amp; 36 h AgNO₃</td>
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<td>36 h fixative &amp; 24 h AgNO₃</td>
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- = absent; + = few; ++ = moderate; +++ = enormous
contrast, because of more background, the camera lucida tracings were difficult and spine details were not prominently visible in the rapid-Golgi stained sections (Fig. 2G and 3G).

There was no significant difference in the dendritic spine densities of both Purkinje (t=1.579, \( P = 0.116 \)) and pyramidal neurons (t=1.125, \( P = 0.262 \)) when compared between the rapid Golgi and Quick Golgi

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**Fig. 1**—Photomicrographs showing rapid Golgi stained cerebellar sagittal sections of P-PBS and P-RGF group (n=3). [Green arrows indicate the Golgi stained fibrous astrocytes, blue arrows indicates Purkinje neurons, pink arrows depicts Golgi stained Bergmann glial fibers. P-RGF: perfusion with rapid-Golgi fixative solution, P-PBS: perfusion with phosphate buffer saline only. Scale bar = 200 µm.]
Fig. 2—Light microscopic images of the Rapid-Golgi (A, C, E) and Quick Golgi stained (B, D, F) cerebellar sections, showing a contrasting difference in the clarity of information in Quick Golgi stained preparations. [Red arrows represents the Golgi stained blood vessels. Scale bar =100 µm.]
Fig. 3—Photomicrographs showing the rapid-Golgi (A, C and E) and Quick Golgi (B, D and F) stained cortical sections. [Red arrows indicate the Golgi stained blood vessels (BV). Pyramidal neurons with their processes are clearly visible with both the methods, but with better cellular details in Quick Golgi method due to reduced staining of the blood vessels. Camera Lucida drawings (1000X) of the main shaft (MS) and primary branch (PB) of apical dendrite showing the better details of dendritic spines with Quick Golgi procedure (H) than rapid Golgi (G). Scale bar =100 µm.]
methods, suggesting that the present modification improves in the qualitative visualization of spine without compromise in quantitative evaluation (Fig. 4).

The proposed Quick Golgi method involves the following steps:
1. Anesthetize the animal (rat) with ether vapors.
2. Perfuse transcardially with 200 mL of PBS followed by 100 mL of rapid-Golgi fixative solution.
3. Dissect out the brain tissue of interest and cut tissue blocks of 1 cm\(^3\) (or smaller) and post-fix in the rapid-Golgi fixative for 36 h.
4. Rinse briefly with 2-3 changes of 0.75% aqueous AgNO\(_3\), in dark.
5. Immerse tissue blocks in 0.75% aqueous AgNO\(_3\) solution for 36 h in dark amber coloured bottle.
6. Remove any precipitate by rinsing with 70% alcohol and then store in 70% alcohol.
7. Cut vibratome sections of 100 µm thickness and collect in 70% alcohol.
8. Dehydrate in 90% and absolute alcohol, clear in xylene and mount in DPX.

**Discussion**

The importance of Cajal’s Golgi method in visualization and study of dendritic spines has been well reviewed by Garcia-Lopez\(^3\). Rapid-Golgi method is frequently used to study the morphology of neurons, glia and dendritic spines in brain sections. This technique is also widely used and a very useful tool in neuroanatomy, CNS pathology and in estimating the functional status of a brain region under study in terms of neuronal processes and spines in them\(^8,9,29\). Dendritic spines are the earliest to be affected in most neurodegenerative disorders like Alzheimer’s disease (AD), Parkinson’s disease (PD), schizophrenia and depression\(^34-36\). The changes are especially related to the number, size, shape and origin in the neurons. The available software (computer program) and hardware (microscope and related software) have made study of spines easy and allowed detail study of such structures\(^29,37,38\). Even the biochemist and molecular biologist can benefit from this method, as it is easy, procedure is simple, and requires minimal instrumentation.

Cajal in 1887 modified the original Golgi method introduced in 1873 by Golgi. It is known as rapid-Golgi method and has been variously modified largely based on the type of tissue available (live, post-mortal, preserved, size, species, etc.) and each modification has led to a different labeling pattern\(^2,3,33,39\). The modifications validated and are in use are many but to list a few: the rapid Golgi procedure, the Golgi-Cox technique\(^40,41\), the Golgi-Kopsch method\(^42\); the Del Rio-Hortega method\(^43\) and others.

Most of these variations of original Golgi method are based on the two step procedure that label the cell profiles; first, the tissue blocks are immersed in the chromating solution (either potassium chromate and/or potassium dichromate) and in the next step the tissue blocks are immersed in silver nitrate that produces silver chromate crystals, that allows the labeling of neurons and other cellular inclusions of the brain. Other variants replace the silver nitrate with mercuric chloride and so on.

The major issues with the rapid Golgi method proposed for modifications are: it takes several weeks to get results due to long fixation time, the tissue becomes brittle that causes problem in tissue sectioning\(^32\); stained particles of blood vessels in the vicinity of stained neurons and glial cells that interferes in visualizing and capturing images of stained cells.

In the present modification the issues like time, elimination of tissue shrinkage, sectioning, staining of cellular structures like fine branches of neuronal processes, uniform clear background, reduced staining of blood vessels, etc. have been addressed. In addition to an earlier modification including use of a vibratome for sectioning that needs no embedding, no expensive cryostats, ease of cutting sections with a sharp razor blade, no need of cryopreservation and less tissue damage due to fragmentation\(^29\) have been proposed.

This modification is novel in the sense that for the first time perfusion of animals with rapid-Golgi...
fixative solution instead of the immersion fixation or perfusion with PBS alone before immersion fixation has been tried. This promotes uniform penetration and thus impregnation of the rapid-Golgi fixative (chromating solution), much clearer background, reduced fragments of precipitate and crystals, uniform impregnation of the chromating solution, better and homogenous formation of silver chromate thus better staining of the neurons, interneurons and glial cells. This also has the advantages of the vibratomy as well as better impregnation of the Golgi solution at the body temperature of $37 \pm 1 \, ^{\circ}C$ and enables to get preparation ready for study in just 3 days.

The present modification not only have made the process quicker but also is easy even for the researchers without an exposure to neuroanatomy, such as biochemists and molecular biologists, who can benefit from this simple method that needs no special infrastructure/instrumentations.

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