Microscopic Anatomy

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Review

The unending fascination with the Golgi method

Y Koyama*

Abstract

Introduction

In the second half of the 19th century, Camillo Golgi provided a breakthrough staining technique for visualising whole neurons, which are seen as black bodies due to intracellular staining with microcrystalline silver chromate. The high contrast, selective staining properties enabled identification of complete neuronal morphology. This staining technique, termed the Golgi method, was later improved by Ramón y Cajal and popularised through his tireless experiments. Morphological analysis, using the Golgi method, led to the discovery of neuronal microstructures such as dendritic spines and growth cones and helped give rise to the 'neuron doctrine'. Many post-mortem human brains as well as brains of experimental animals have since been stained using this method. In combination with other morphological techniques (e.g. electron microscopy and immunohistochemistry), the Golgi method has allowed us to glean more information regarding the neuronal networks present in various brain regions. However, the Golgi method is a difficult first choice for morphological analysis since it is capricious, complicated and time-consuming and has poor reproducibility.

Recent increases in the number of in vivo animal experiments and of post-mortem brains collected following neurological disorders heighten the need for the Golgi method to be viewed as a crucial morphological tool for assessing abnormalities in single neurons, as well as in neuronal networks. Fortunately, over 100 years of neuroanatomical diligence has seen significant contributions to overcoming the shortfalls of this method. The advent of modified Golgi methods with potential use as routine techniques, together with the development of the kit-based Golgi–Cox method, has made the Golgi method more accessible to neuroanatomists.

This review surveys the technical fundamentals, history and evolution of Golgi methods and intends to spark an interest in the Golgi method within every neuroscientist, novice and old pro alike and to allow them to appreciate this useful technique.

Conclusion

Many neuroanatomists, including us, feel a strong attraction to the Golgi method as a powerful morphological tool. Our researchers have identified unwanted issues of the various Golgi methods and then have been working to remedy these problems. We encourage the reader to adopt staining using the Golgi method as its utility continues to evolve.

* Corresponding Author
E-mail: koyama@anat2.med.osaka-u.ac.jp

Department of Anatomy and Neuroscience, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan

Introduction

While studying the brain function, it is extremely important to investigate the precise shape and morphological changes of individual neurons. A neuron is a specialised cell that emits an electrical signal to allow information exchange, a characteristic not found in the cells of other organs. Both the short 'dendrite', which is intricately branched like a tree branch and the long 'axon' emanate from the nerve cell body. In addition, neuronal spines located on dendrites receive electric signals from other neurons and are involved in neuronal plasticity. Thus, together with neural cells such as neuroglia, neurons form complicated networks known as 'neural circuits'.

To appreciate the complexity of such intricate neural networks, staining methods that allow the visualisation of neuronal cells in thinly sliced brain sections are used. In particular, Nissl stains and silver impregnation are commonly used. Nissl staining, which is based on a mechanism combining a basic aniline dye with Nissl granules, can stain both the cell nucleoli and rough endoplasmic reticula in neurons. In contrast, silver impregnation using neuronal argent affinity can stain an entire neuron, but not the myelin sheath. Neural circuitry refers to the combination of many interacting neural cells and is immensely complex morphologically, with many neurons intertwined with one another within a restricted space. Unfortunately, because the above techniques stain all neural cells with equal probability, it is difficult to identify and appreciate the morphology of a single cell amongst the mass of other stained cells.

In contrast, the Golgi method, focused in this review, has allowed for the visualisation of entire neurons and glia in high detail and with good contrast. Moreover, compared to Nissl staining and silver impregnation, the Golgi method has the beneficial feature of characteristically selective staining. Because neurons are stained only sparsely with the Golgi method, it is a powerful staining technique for providing a complete, detailed representation of a single neuron. The aim of this review was to discuss the history and evolution of the Golgi method.

Discovery and popularisation of the Golgi method

In the late 19th century, the Italian scientist Camillo Golgi discovered an epoch-making staining technique.

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After preparing brain sections of 4–5 mm thickness, the brain sections were hardened with potassium dichromate and then immersed in silver nitrate[1]. As a result, the neurons were stained darkly with silver chromate crystal that had incorporated into them. Golgi called this 'reazione nera' and successfully developed a staining technique for visualisation of the whole neuron. Subsequently, this staining technique was referred to as the Golgi method.

The Spanish neuroanatomist Santiago Ramón y Cajal was impressed by Golgi's staining and sought to use it in his neuroanatomical studies[2]. He modified the original Golgi method by adding osmium tetraoxide to the potassium dichromate solution. Thereafter, unique neuronal projection structures (e.g. dendritic spines, growth cone and filopodia) were discovered through morphological analysis of various brain specimens stained with this modified Golgi method, which was termed the Rapid Golgi method. The excellent results produced with this technique exposed the Golgi method to the wider neuroanatomical world.

In 1906, Golgi and Cajal jointly received the Nobel Prize in Physiology or Medicine for their achievement in elucidating various aspects of the nervous system structure[3]. Unfortunately, their theories on the mechanisms of neural network function differed. Golgi agreed with the mainstream conception postulated by Gerlach, the reticular theory of the nervous system, in which neuronal networks constituted a single neural syncytium. In contrast, Cajal put forward the neuron doctrine of the nervous system, in which many distinct, individual neurons interacted to form a neuronal network[4]. Since that time, both the invention of electron microscopy (EM) and the discovery of the synapse have demonstrated that neuronal networks comprise individual neuronal cells. Many post-mortem human and animal brains have since been stained using Golgi methods[5–7]. These studies, using the method developed by Golgi and popularised by Cajal, have disproportionately contributed to the study of neuronal morphology.

Mechanism of the Golgi method

In the Golgi method, it is possible to stain various neural cells such as neurons and neuroglia, as well as cells from the vascular system. The crystal of the silver chromate taken up by neurons spreads into every corner of the neuron. Moreover, the Golgi method provides images of neurons as dark features on a transparent background with good contrast. Therefore, the method enabled the discovery of previously unknown neuronal structures such as growth cones, dendritic spines and filopodia.

As mentioned above, the Golgi method has an exclusive property – its capricious selectivity. As a potential mechanism underlying such selectivity, Shankaranarayana et al.[8] speculated that the stainability of an individual cell might be significantly affected by interaction between the cellular status during fixation (e.g. metabolic state and pH) and the heavy metal used for staining. We have recently acquired evidence to support this assumption. Our report demonstrated that a modified Golgi method used for staining primary cultured neurons did not possess selectivity, typical of the Golgi technique. The underlying reason was believed to be that the bioactivity of each cultured neuron was extremely uniform due to the re-initialisation of the hippocampal neuronal cells after harvesting in the culture medium. However, the precise mechanism of selective stainability remains to be confirmed. The unique selective stainability of the Golgi method has allowed visualisation of the entire morphology of single neurons. This has enabled researchers to determine the projection sites as well as the morphology of neuronal cells.

Traditional methods of analysis applied to Golgi-stained samples included observational study using light microscopy or measurements provided by camera lucid drawings. A great deal of information is available from analysing such photos and images, including the total length of neuronal processes, number of branch points, complexity of the dendritic tree and types of dendritic spines[7]. Further, recent improvements in microscopy and computer software have allowed high-quality images to be obtained and analysed in three dimensions[9,10]. The accumulation of such information has led not only to the revelation of detailed morphological aspects of neural circuitry in various parts of the brain, but also to information on abnormal neuronal morphologies encountered in brains with neuronal diseases.

Various modified Golgi methods

The staining in which brain tissue is penetrated by dichromic acid is generally called the Golgi method. There are three major classes of Golgi method: Rapid Golgi, Golgi–Kopsch and Golgi–Cox.

Rapid Golgi method

The staining provided by the original Golgi method was not reproducible because the unstable precipitate of chromate silver and lipoprotein is limited to the cell membrane. Therefore, in the Rapid Golgi method developed by Cajal, the staining was improved by adding osmium tetraoxide to the original potassium dichromate solution[3]. Because osmium stabilised cell membranes, permeation occurred perfectly and promoted efficient crystal formation, allowing the staining of numerous neural cells[11,12].

Although the Fox-modified method to stain formalin-fixed brains and cerebral tissue was useful in

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studying adult mice[13], the Rapid Golgi method was initially believed to act best with fresh brains[14] or on short-term formalin-fixed brain samples[15,16]. It was later recognised that this technique was also suitable for staining tissues fixed with formalin[17–19]. Moreover, using the Rapid Golgi method, samples of older animals stained better than those of younger animals[20].

There are some variants of the Rapid Golgi method. For example, Hortega Rio[21] modified the Rapid Golgi method by using formalin together with a dichromate salt and chloral hydrate. Scheibel et al.[22] investigated glial cells and cerebellar granular cells using this modified method.

Golgi–Kopsch method

Kopsch[23] devised the Golgi–Kopsch method, which uses formaldehyde instead of osmium. This method allows animals of any age and any region of the brain to be studied[20]. In 1964, the Clonnier–Golgi method, invented by Clonnier[24], used glutaraldehyde (GA) as a substitute for osmium. Collectively, these two methods are also known as the aldehyde Golgi method.

This method can stain brain samples to be kept fixed for several years[17]. Moreover, it was possible to stain animal samples immediately after perfusion and human brains soon after removal from the head[20,25]. However, the number of impregnated neurons is fewer in the Golgi–Kopsch method compared to the other two methods. To overcome this, a modified Golgi–Kopsch method was designed to increase the number of stained neurons by adding triton X-100[26].

Golgi–Cox method

The Golgi–Cox method, devised by Cox[27], involves tissue samples being immersed in a mixture of potassium dichromate and mercuric chloride. Since the background staining is less intense than for the Rapid Golgi method, the Golgi–Cox method is best suited for analysing the complete dendritic tree of cortical neurons[15]. Of the three Golgi methods, the Golgi–Cox method is the optimal method for examining the different types of neurons present in adulthood. However, invasion of neuronal spines is poor[15,28] and hence this method is not optimal for investigating subcortical structures[19]. In addition, it has been reported that Golgi–Cox stained samples tend to contract more than the samples stained using the Rapid Golgi technique[29].

As pointed above, each method has drawbacks and advantages. Given below is a concrete example. Regarding the preservation state and stainability of a specimen, both the Golgi–Cox and Rapid Golgi methods are able to stain many neuronal cells in unfixed and short-term fixed specimens (Cox: up to the 7th day of fixation; Rapid: up to at least three and a half months of fixation)[30]. However, the staining provided by the Golgi–Kopsch method for such samples is inferior to that obtained with the two other methods. In contrast, the Golgi–Kopsch method allows for vivid staining of fixed samples from specimens aged 15 months to 55 years and the staining for long-term fixed tissues is superior than that with the other two methods[30]. Therefore, the choice of method is best determined according to the desired tissue preservation (i.e. fresh, short-time fix, long-term fix), the age of the specimen, the research object of interest (e.g. dendritic tree and neuronal spines) and so on.

Technical applications of the Golgi method

The Golgi method has been combined with various techniques for morphological analysis in order to capture as much information as possible about individual neural cells and the neural circuits in which they reside.

Tracer injection and immunohistochemistry

Somogyi and Smith et al.[31,32] reported that the Golgi method can be performed after injection of a horseradish peroxidase (HRP) tracer because HRP itself does not deteriorate during immersion; it is detectable by immunohistochemistry (IHC) in Golgi-stained brains. This facilitated our understanding of the positional relationship of the stained neuron within the neuronal network. Further, Somogyi et al.[33,34] performed EM analysis of specimens stained using a combination of the Golgi method and HRP injection. A combined Golgi–IHC method for performing IHC after de-impregnation of chromate compounds has also been reported[35]. Unfortunately, these methods have little practical value for use as routine methods because of their complicated nature and the inconsistent results produced. However, the development of confocal laser scanning microscopy (CLSM) has been a watershed in the history of the Golgi–IHC method. Images of Golgi-stained neurons were reconstructed with CLSM using a method called the reflection method[9]. High contrast images were obtained due to a computer-mediated digital reduction of background noise. This method provides an advantage in that it enables investigation of both the type of stained neuron and the environment around the neuron, due to immunofluorescence staining that can vary according to the neuronal marker used[10].

Electron microscopy

In 1965, Blackstad[36] and Stell[37] advocated that Golgi-stained samples be investigated with EM. Blackstad et al.[38] investigated the immersion substrates used by the Golgi method, namely chromium silver, under EM. The particular Golgi method used in this combined Golgi–EM technique was the Rapid Golgi method[39] rather than the Golgi–Cox method[40]. Both the use of silver and fixation with

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aldehyde were cited as the reasons for this choice[17]. Since fixation could minimise damage of the tissues and silver exhibited enough stability under electron-beam irradiation to use for immunoelectron microscopic analysis, the Rapid Golgi method was thought to be better suited for the combined Golgi–EM study. Fairen et al.[41] were most successful in using the combined Golgi–EM method, having replaced the chromate silver with gold to enable the cell organelles and synaptic regions to be clearly visualised under EM. Thus, the combined Golgi–EM method is a useful morphological tool for providing much more detailed anatomical information than light microscopic analysis.

Problems with the Golgi staining method

Since the invention of the Golgi method, this powerful tracing technique has contributed greatly to our understanding of various neural circuits. However, other techniques such as HRP tracer injection and immunofluorescence have often been used in place of the Golgi method. Below, we outline some of the reasons for this.

For one thing, some difficulties had arisen when Golgi methods were employed. Though chromatinisation is an important process in all Golgi methods, it takes a long time and must be handled with care. Increasing the reaction time for chromatinisation causes performance deterioration of cutting the brain samples resulting from tissue hardening[30]. Increasing the time for impregnation and failing to change the bathing solution results in a reactant of bichromate and aldehyde[24,42,43]. Such precipitation leads to non-specific staining, obscuring the staining of the desired cell[30].

Alternatively, some reports have suggested that the impregnation of chromate compounds in the Golgi method is vital[44,45]. However, many reports have indicated difficulty in obtaining reproducible results due to unstable staining[15,16]. In addition, the laborious nature of the procedure prevents the Golgi method from being viewed as a first choice for morphological analysis.

Additionally, the characteristics of the Golgi method may be unsuitable for specific research questions. For example, difficulty in staining nerve plexuses has been observed[46] and axial fibres of adult myelinated nerve cells cannot be stained since the crystal cannot permeate the myelin sheath[47]. Moreover, the capricious staining observed with the Golgi method limits the number of surveyed neurons, since only ~5% or less of neuronal cells are stained[48]. In short, the Golgi method is unsuitable for examination of adult axons or in cases where the desire is to stain many cells. Thus, alternative, reproducible and more convenient techniques have become preferred to the Golgi method.

Vigorous efforts to overcome the challenges facing the Golgi method

In recent years, as research using experimental animals (e.g. disease-model animals and knockout animals) and human post-mortem brains with neurological symptoms has become more common, various morphological techniques have been required to compare brains of model animals or human brains with neurological diseases with control samples, to identify the site of abnormal morphological changes. Although some disadvantages in the Golgi method exist, as described previously, this and other techniques (e.g. Nissl staining and tracer injection) have been used to investigate morphological alterations from diverse standpoints, resulting in the collection of large amounts of information. Therefore, many neuroanatomists have tried to improve the Golgi method by reducing impregnation time, simplifying the process to achieve and an increase in the number of stained neuronal cells. Various treatments or requirement study of impregnation have been carried out in the following ways: vacuum treatment[6], examination of temperature[43,49,50], investigation of pH[25,51,52], application of brain samples coated with egg yolk[53], changes in the tissue embedding media[54,55], auto metallographic enhancement[56], usage of vibratome[57], use of single sections for staining[58] and so on. Microwave irradiation was considered to be a particularly effective treatment. Microwave irradiation improved the staining obtained with the Golgi–Kopsch[59] and Rio-Hortega Golgi method[60], facilitating crystal growth. Alternatively, in the Golgi–Cox and Rapid Golgi methods, although glial cells were well-stained by microwave irradiation, the staining of neurons was reduced[61]. Thus, by the varying improvements seen with different treatments, each modified method contributes to solve the problems associated with the Golgi method. Indeed, this is reflected by the fact that the Golgi method has become increasingly easy to use year after year.

There are three interesting papers of late containing details of relatively useful modified Golgi methods and Golgi combined methods. One is the Golgi–Cox method as modified by Amit Ranjan et al.[62], which involved staining times as short as 48 hours and enabled separate staining of cells by including or not including fix treatment (non-fix: neurons, formalin-fix: glial cells). The second adaptation is to the Rapid Golgi method, which enabled good contrast images to be obtained and the staining to be complete within the space of three days[63]. The third paper describes the advanced Golgi–Cox method devised by Nathan et al.[64], which was also applied to samples treated with various fixatives (e.g. paraformaldehyde, glutaraldehyde and acrolein) and reduced the impregnation time due to the use of 60–100 μm thin sections.

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Moreover, because the thin sections were produced using a vibratome, alternate sections could be analysed using complementary techniques such as immunohistochemistry and electron microscopy.

Contrastingly, the invention of the FD Rapid GolgiStain Kit (FD NeuroTechnologies), in 2002, to address several of the difficulties associated with the Golgi method was a welcome relief for neuroanatomists. The kit was developed based on Golgi–Cox methods[28,65]. The use of the kit, as directed, enables every researcher, including those not savvy with the Golgi method, to investigate the detailed morphology of neurons as well as to obtain an overview of neural networks in brains from perinatal periods to adulthood. Moreover, our improved kit-staining methods allowed for the staining of foetal brains by including fixative treatment[66]. Further, we succeeded in applying kit-staining samples not only to standard EM but also to ultrahigh voltage electron microscopy[67]. Therefore, observational study using the kit-stained samples now provides more choices than ever before. Collectively, recently reported Golgi method variants and the advent of the Golgi kit have made the Golgi method accessible to a much greater number of researchers.

Discussion

The author has referenced some of his own studies in this review. The protocols of these studies have been approved by the relevant ethics committees related to the institution in which they were performed. Animal care was in accordance with the institution guidelines.

Golgi method was an important technique for morphological analysis of in vivo study. Although this method was allowed for the visualisation of whole neurons, it is difficult to use it routinely because of the complicated procedure, the unpredictably of staining and the long time involved. Fortunately, the problem of Golgi method is on the way to be solved thanks to a lot of neuroanatomical diligence. We think that fixed treatment is the key to improving the stainability of the Golgi method. Addition of osmium, aldehyde and glutaraldehyde altered the stainability dramatically because the stabilisation of cell membranes by fixture enabled many silver chromate crystals to penetrate and remain in neurons[11,12,23,24]. In addition, our experiment also revealed that the aldehyde fixation of specimens before impregnation increased the number of stained neurons in the Golgi–Cox method[66]. In particular, the mixture of paraformaldehyde and glutaraldehyde (aka; Karnovsky's fixative[69]), which is a common fixative used for microscopic anatomy, proved to be effective. Because the enhancement of stainability led to stable results, the fixture was essential for the development of the Golgi method.

Finally, the Golgi method went beyond the bounds of in vivo staining technique. Following animal studies, primary neuronal cultures were often prepared to examine the action and function of a target molecule identified using an in vivo experiment, to assess its effects on neurons. When analysing neural circuits using the Golgi method, we happened to discover that there was no Golgi method appropriate for staining primary cultured neurons. We, therefore, reported the successful development of a modified Golgi–Cox method adaptable to primary cultured neurons, in which the amount of mercury compound incorporated into neurons was increased by fixation with aldehyde and rapid freezing[69]. Further, visualisation was improved by using a fluorescent antibody (Figure 1). This method was equal in sensitivity and resolution to analysis using recombinant neurons expressing green fluorescent protein and enabled staining of the entire length of cultured neurites, including growth cones and neural spines, regardless of the time for in vitro culturing. We believe that such emerging technologies based on the Golgi method will continue to be developed and will prove increasingly helpful for unravelling neuronal circuits from a different vantage point.

Conclusion

The Golgi method is a very impressive technique not only for investigating the entire morphology of an individual neuronal cell but also for investigating neural circuits. Unfortunately, it has long been unsuitable for routine use because of the complicated procedure involved, the unpredictably of staining and the large amount of time that must be invested. However, with the increasing importance of in vivo studies, we reaffirm the need to analyse the morphology of neurons and neural circuits using the Golgi method. In addition, the Golgi method has also been reported to be a significant tool for the examination of brain plasticity in mental processes.

Therefore, many neuroanatomists, including us, feel a strong attraction to the Golgi method as a powerful morphological tool. Our researchers have identified unwanted issues of the various Golgi methods and then have been working to remedy these problems. We encourage the reader to adopt staining using the Golgi method as its utility continues to evolve.

Abbreviations list

CLSM, confocal laser scanning microscopy; HRP, horseradish peroxidise; IHC, immunohistochemistry.

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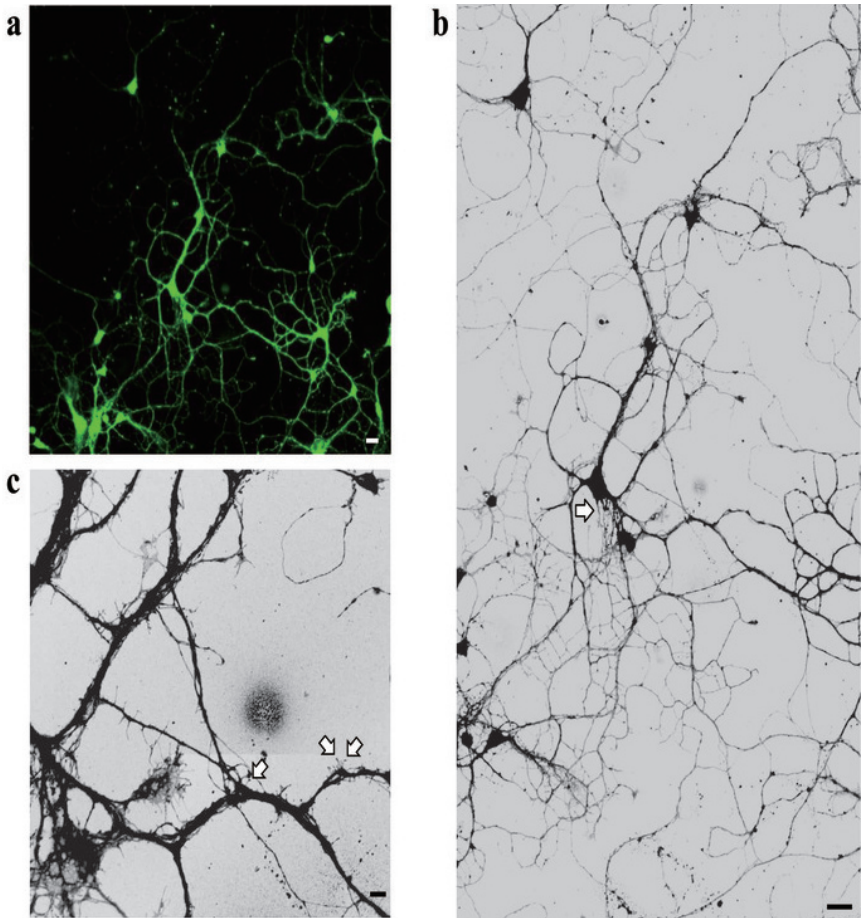

Figure 1: The fluorescent Golgi method allows for visualisation of an entire single cultured neuronal cell, including its axon, dendritic tree and spines. (a) RGM colour image and (b, c) gray-scale image. (a) Low-magnification image, (b) photomontage consisting of high-magnification images and (c) dendritic spins. The arrows indicate (b) axon or (c) spines. Scale bars (a, b) 20 μm and (c) 1 μm.

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