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GOLGI METHOD: A 140 YEARS OLD YET UNIQUE AND POWERFUL METHOD FOR THE STUDY OF THE CENTRAL NERVOUS SYSTEM

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Abstract

Golgi method is been using for more than 140 years so far for the study of the individual morphological and morphometric characteristics and parameters of the cells of the central nervous system. Although other methods came to light, Golgi method is still unique and one of the most powerful tools in the hands of the neuroscientists. What makes Golgi method unique is the capacity to stain all components of the brain tissue, including neurons, glial cells and the vasculature. The cell soma, the dendritic arborization including the spines and at least a part of the axon are usually visible, providing a panoramic view of the entire neural element. Golgi method can be combined with modern and sophisticated techniques which can reduce the human interference and produce accurate 3D models of the neuronal elements of the central nervous system.

Key words*:* Golgi method, 3D Neuronal Reconstruction, Neuromorphology.

Introduction

The modern scientific investigation of nervous systems started over a century ago with the revolutionary neuron doctrine, posted by Santiago Ramon y Cajal. Cajal showed that, like all the other organs in the body, the brain is constituted by cells and revealed the incredible complexity of the shape and potential connectivity of brain cells. Cajal's findings inspired the principal axiom of modern neuroscience: the key substrate for all the functions performed by nervous systems, from regulation of vital states, reflexes, and motor control, to the storage and retrieval of memories and appreciation of artistic beauty, lies in the structure and assembly of neurons (Mavroudis and Alexiou 2015).

The ultimate, and arguably the hardest, challenge to human knowledge consists of understanding how neurons and their connections give rise to feelings, emotions, and logical thinking.

One of the most powerful methods for the study of the neuronal structure of the central nervous system is the 140 years old yet unique Golgi method.

Golgi method

More than 130 years ago, Camillo Golgi introduced a staining technique for visualizing whole neurons, which are stained black or dark brown, on a yelowish-golden background. Camillo Golgi in his original work used silver nitrate and potassium dichromate; however his method was not reproducible because of the unstable nature of the precipitate of chromate silver on the lipoprotein of cell membrane. Santiago Ramon y Cajal improved Golgi's original method, adding osmium tetraoxide to the potassium dichromate solution, which is stabilizing cell membranes, allowing the visualization of more neurons (Rapid Golgi method). Working with his modification of Golgi method Cajal described for first time the neuronal cells as distinct entities and visualized dendritic spines and growth cones. Several modifications of Golgi method have been developed so far, which all have in common the impregnation of neuronal cytoplasm with metallic salts (Baloyannis, et al. 2011) (Raju, et al. 2004). The most important Golgi modifications are the Golgi-Cox method, which was developed by Cox and is particulalry useful for tracing dendritic arborization, the method which was developed by Hortega del Rio using formalin with the dichromate salt and chloral hydrate, and which is useful for the study of neuroglia, small granule cells and the cerebellum, and finally the Golgi-Fox method, which was first introduced by Fox and is useful for adult formalin fixed brain tissue (Das, Reuhl and Zhou 2013).

After the development of intracellular labeling techniques using horseradish peroxidase and biocytin, Golgi methods have taken second place, yet the new methods never came close to matching the overview of entire brain areas that Golgi methods can provide. Positively even after 130 years Golgi technique is increasingly used for qualitative histology and neuromorphology, neurobiology, experimental neurology and neuropathology (Raju, et al. 2004) (Overdijk, et al. 1978).

What makes Golgi method unique is the capacity to stain all components of the brain tissue, including neurons, glial cells and the vasculature. The

percentage of the neurons that are impregnated varies from 1 to 10%. The cell soma, the dendritic arborization including the spines and at least a part of the axon are usually visible, providing a panoramic view of the entire neural element (Glaser and van der Loos 1965)(Fig. **1**).

The basic steps of Golgi method include:

1. The selection of brain area that will be studied
2. Immersion in formaline solution for more than 25 days
3. Fixation in potassium dichromate solution
4. Immersion in silver nitrate aquous solution
5. Fixation in alcohol
6. Cut in thick sections of 100-120μm
7. Study in a light microscope

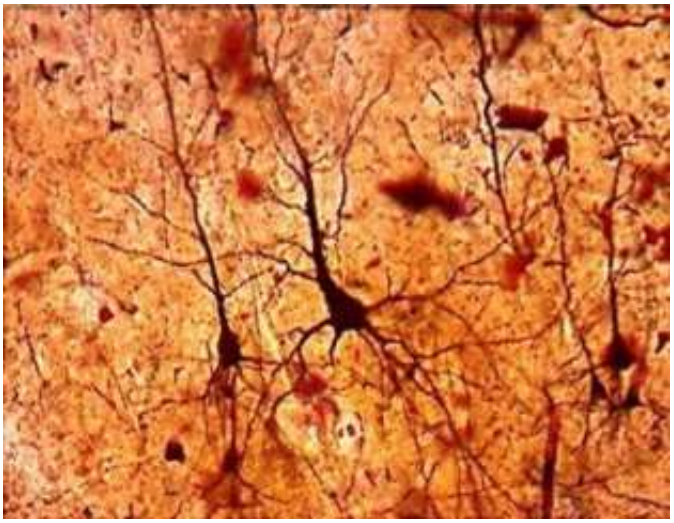


Fig. 1: Golgi stained Pyramidal neurons from the human visual cortex. Magnification 100X

Although Golgi method has been using for more than 140 years now, there are many questions that have to be answered. The studies of Blackstad (Blackstad 1958) and Stell (Stell 1965) showed that the main chemical reaction is that of the creation of a lipoprotein-chrome-nitric compound.

It is not easy for one to provide safe guidance and instructions for the success of the method, however one of the most important factors is the condition of the tissue before the staining process, and in general the intensity of the stain on human dervived material seems to be related to the age of the person, the study area (cortical vs subcortical areas) and the overall fixation process.

Any effort on identifying environmental factors that could contribute to the improvement of the quality of Golgi method had no clear results so far, but after many years of experience and observation in the laboratory of Neuropathology of the 1st Department of Neurology of the Aristotle University of Thessaloniki, we have concluded that a temperature between 18-22ºC showed the best results and a fixation time of 3-7 days in the potassium dichromate solution and 2-7 days in the silver nitrate, depending on the concentration of solutions ranging from 2.66 to 3% and between 0.75-2% respectively. Additional observations led us to the addition of formaldehyde solution 37% 1ml per 100ml of potassium dichromate solution, which probably contributes to the building of bridges between cysteine residues of proteins of the cell membrane, and the lipoproteino-chrome-nitrate cluster, thus staining a larger number of neurons reducing in parallel the chromic artefact deposition.

Neuronal staining starts from the cell body, and then the apical dendrite and the basal dendrites are following with the difference of staining speed to be related to the protein concentration in every different part of the dendritic tree. The next step in the the tracing of Golgi stained neurons which will provide accurate 3D models and will extract the investigated morphological parameters.

Neuronal Tracing

The first and critical task in the study of neuronal morphology is the selection of neurons, which will be traced. The selection of neurons is based on the criteria set forth by Jacobs and requires that all quantified neurons should appear fully impregnated and possessed relatively complete, uninterrupted basilar dendritic systems, consisting of at least three primary dendritic branches, and subsequent higher-order branching (Jacobs, Driscoll and Schall 1997).

Neuronal tracing and reproduction of an accurate 3D model of a neuron can be done either with manual, semi manual, semi-automatic or completely automatic methods with the help of specific software commercial or freeware, Neurolucida and MicroBrightField or Neuromantic, NeuroMorpho and NeuronStudio respectively.

In contrast with early approaches to neuron tracing using specialized computer controlled microscope systems, which stored only the morphological features measured directly from the imaged samples but not the images themselves, the preferred way nowadays is to first acquire the full image data, as it guarantees a permanent record of the original samples and allows the use of

more flexible and more powerful data processing method (Parekh and Ascoli 2013) (Myatt, et al. 2012).

In our method, for each one of the neurons a video of 1 min is recorded, while the microscope table is moving with a stable velocity, so the whole neuron including the dendritic field to be integrated. Then the video is inserted to Image J application, and is analyzed to 120 serial images which are saved as image stack. After uploading the image stack to Neuromantic software the quantification of cell some begins in manual mode, while tracing of dendritic field follows in semi-automatic mode (**Eroare! Fără sursă de referință.**). When the neuronal tracing has been finished full statistical analysis is available and the reconstruction is saved in swc or xml file type. Digital reconstructions enable quantitative analysis of neuronal shapes by means of morphometric parameters describing the metrical and topological properties and the spatial embedding of the three-dimensional structures (Mavroudis and Alexiou 2015). These morphometric parameters make it possible to statistically describe the variability in neuronal morphologies. Three dimensional analysis is completed with Sholl's analysis, which gives the neuronal field density as a function of the distance from cell soma (Sholl 1955).

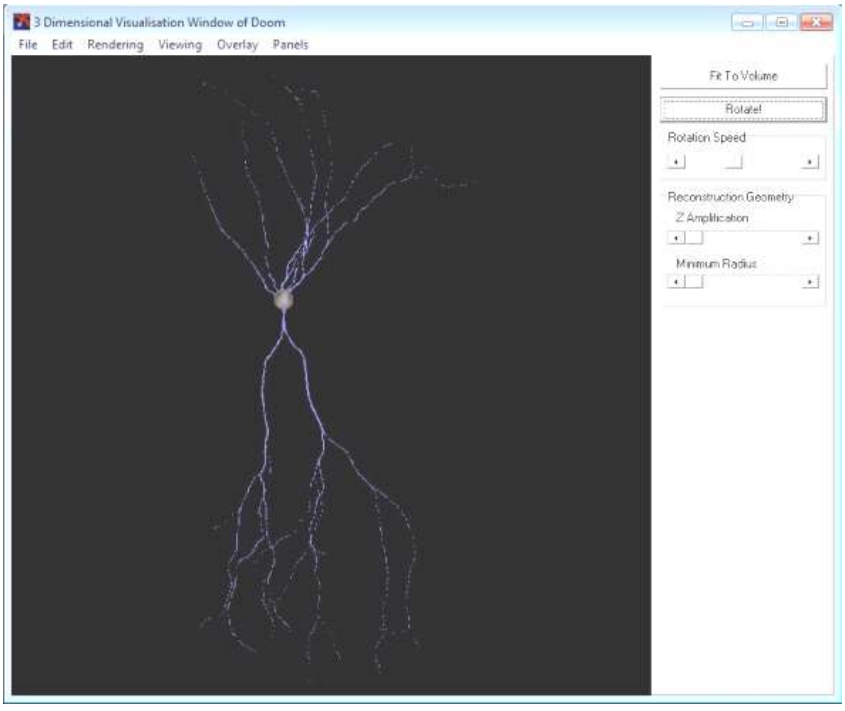


Fig. 2: 3D Visualisation with Neuromantic software of a Pyramidal neuron from the human visual cortex

Sholl dendritic tree analysis

Sholl analysis is a method of quantitative analysis of neuronal dendritic trees, first used to describe the differences in the visual and motor cortices of cats. Initial quantification of a neuron is performed by counting the number of dendrite intersections for concentric circles, usually centered at the centroid of the cell body, of gradually increasing radius (Sholl 1955)(**Fig.**). Curves produced by this initial counting are usually of somewhat irregular shape, and much work has been done to determine appropriate means of analyzing the results.

Neuronal morphometric parameters

The morphological parameters for each traced cell are automatically extracted in a .txt format file (**Fig.**). Although for every traced neuron more than 30 parameters are estimated, the most important of them are the number of stems, which refers to the total number of segments leaving from the dendritic root, the number of branch points which refers to the total number of branch points, the branching orders, which refers to the topological distance from the dendritic root, the total dendritic length which is the summed length of all segments in a tree, the segment length which is the path length of the incoming segments toward a node, the stem length which refers to the path length between a branch point with order =1 and the dendritic root, the number of terminal branches which refers to the total number of terminal branches of the dendritic tree, the Euclidean distance which is used to measure the distance between the soma and the termination points, the neuronal contraction which refers to the Euclidean length of a branch divided by the path length, and finally the Asymmetry of the dendritic tree which refers to the topological complexity of a tree, with completely asymmetric tree having an asymmetry index of 1, and completely symmetric an index of 0 .

Neuronal spines

Appart from dendritic arborization and the morphometric parameters of each one of the neurons, Golgi method also provides excellent images of the dendritic spines, allowing the estimation of their density along the dendritic tree, and the study of the morphological characteristics of them (**Fig.**). Spinal density can be assessed either by semiautomatic tracing with Neuromantic or any equivalent software, or in a more manually defined way on the grounds of multiple images of different segments of the dendritic tree.

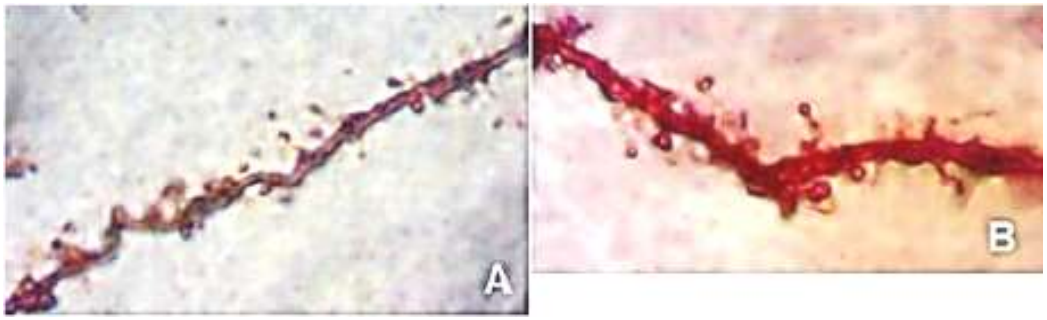


Fig. 3: Dendritic spines as they are demonstrated using Golgi method. Magnification 1000X

Neuronal reconstruction and neuronal function

How neuronal tracing and the study of neuronal morphology can be useful in the understanding of neuronal functionality? From the experimental standpoint, studying the structure–activity relationship in neurons directly is extremely difficult, but computer simulations constitute a powerful alternative. In classical computational models, anatomy is simplified or kept "constant", and the influence of various distributions of active and passive properties on neuronal firing is assessed (Sholl 1955). With a complementary approach, one can keep the biophysical model constant and implement it on different dendritic structures. In this way, investigators characterized the effect of morphological differences among different neuronal classes on their firing patterns and on the dendritic back- and forward-propagation of action potentials (Poirazi and Mel 2001). These findings were recently extended by an analysis of topological influences of firing properties and by studies of the electrophysiological effect of dendritic variability within the same morphological class (van Elburg and van Ooyen 2010).

Model simulation experiments can be carried out in simulation environments like Neuron and Genesis. The NEURON simulation environment can be obtained via the World Wide Web (WWW) at (www.neuron.yale.edu) (**Fig.**). Neuron works on hoc programming language, however a graphic user interface is also available. Neuronal reconstructions can be uploaded and be used for simulation experiments. The user is able to work on passive model or on active models using specific neurophysiological data that are available in the literature (Carnevale and Hines 2008).

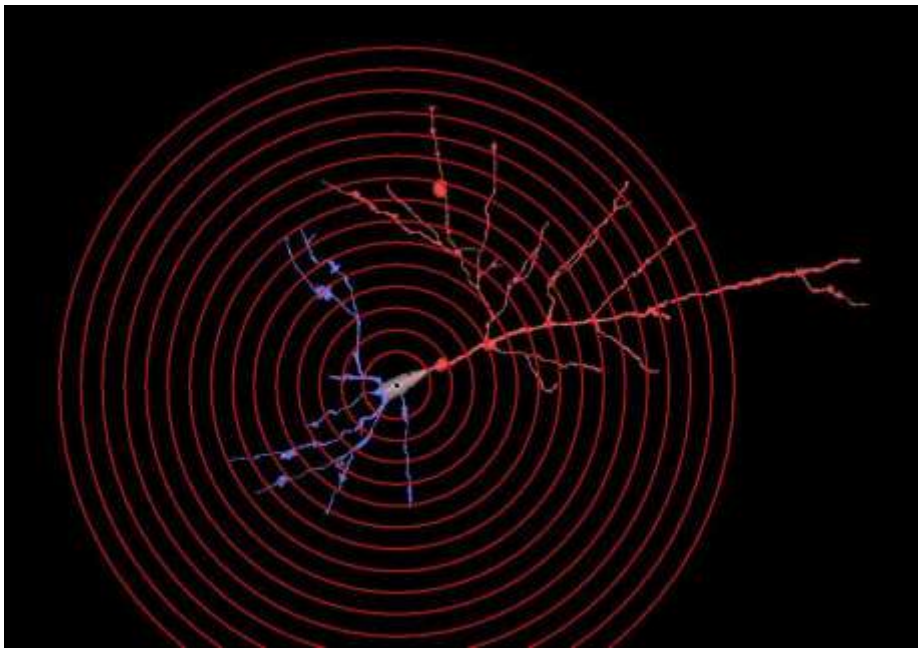
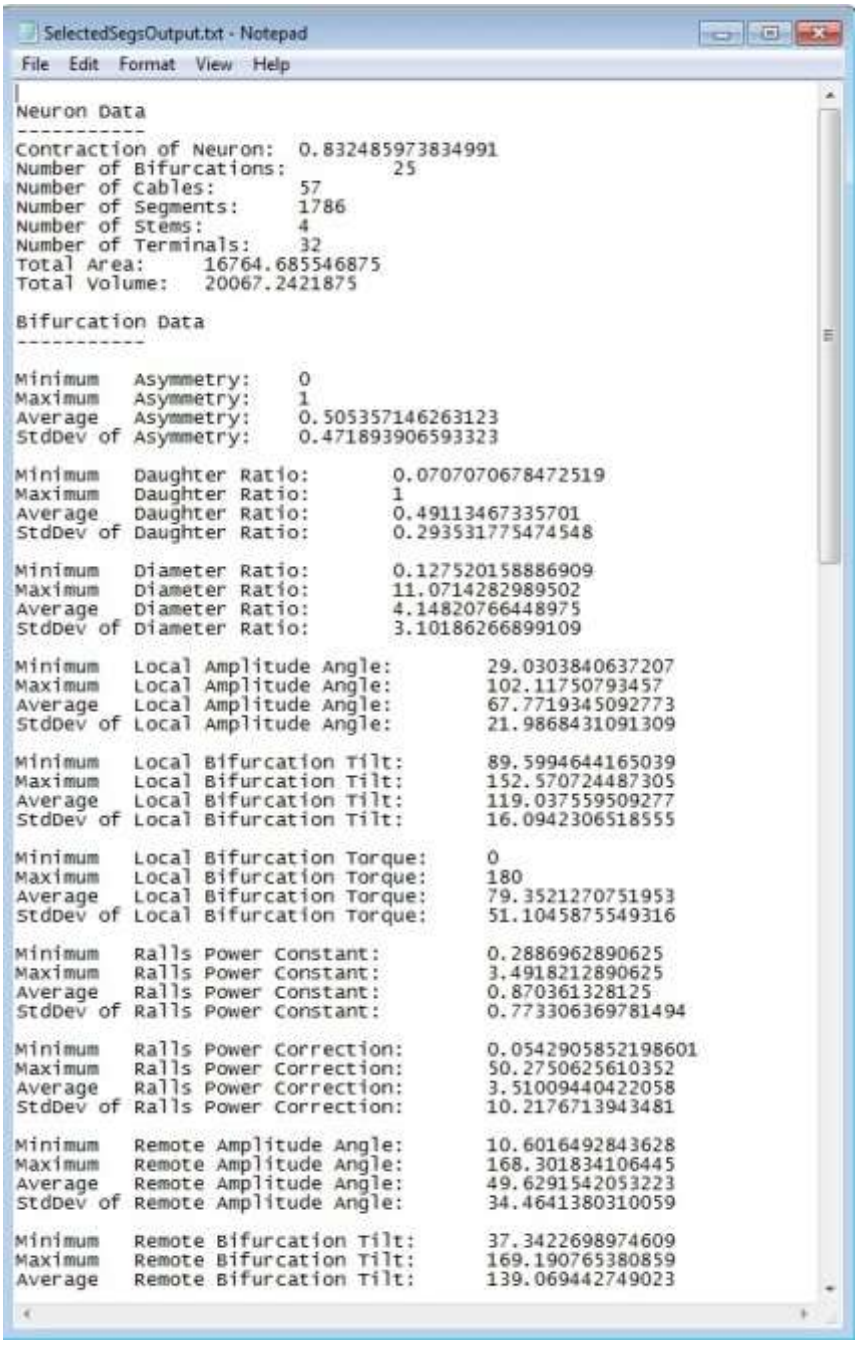


Fig. 4: Sholl analysis is performed by counting the number of dendrite intersections for concentric circles, which are centered at the centroid of the cell body, of gradually increasing radius

Limitations

Neuronal dendrites are not static structures; rather they exhibit dynamic changes that apparently reflect functional changes in the central nervous system. Therefore the data that we obtain from the morphological analysis and 3D reconstruction represent snapshots that may not be entirely representative. Moreover there are numerous practical difficulties in gathering accurate quantitative measurements of neuronal arborisations using conventional light microscopy, namely factors that are related to tissue shrinkage, operator errors, and the limited resolution of the light microscope.

Fixation shrinkage of an entire slice can be assessed by measuring slice size and thickness before and after fixation. Shrinkage factors are estimated from these measurements and applied to the obtained neural reconstructions. These methods assume, however, that shrinkage was uniform throughout the slice and that individual cells shrink at the same rate as the entire slice. This assumption may not hold true in some cases. Shrinkage at the edge of a slice can be different than in the centre, leading to a distortion of cells. Also, individual dendrites may curl up rather than shrink, and curled dendrites may retain their original length (Jaeger 2015).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```
Neuron Data
-----------
Contraction of Neuron:  0.832485973834991
Number of Bifurcations:         25
Number of Cables:       57
Number of Segments:     1786
Number of Stems:        4
Number of Terminals:    32
Total Area:     16764.685546875
Total Volume:   20067.2421875

Bifurcation Data
----------

Minimum   Asymmetry:    0
Maximum   Asymmetry:    1
Average   Asymmetry:    0.505357146263123
StdDev of Asymmetry:    0.471893906593323

Minimum   Daughter Ratio:       0.0707070678472519
Maximum   Daughter Ratio:       1
Average   Daughter Ratio:       0.49113467335701
StdDev of Daughter Ratio:       0.293531775474548

Minimum   Diameter Ratio:       0.127520158886909
Maximum   Diameter Ratio:       11.0714282989502
Average   Diameter Ratio:       4.14820766448975
StdDev of Diameter Ratio:       3.10186266899109

Minimum   Local Amplitude Angle:        29.0303840637207
Maximum   Local Amplitude Angle:        102.11750793457
Average   Local Amplitude Angle:        67.7719345092773
StdDev of Local Amplitude Angle:        21.9868431091309

Minimum   Local Bifurcation Tilt:       89.5994644165039
Maximum   Local Bifurcation Tilt:       152.570724487305
Average   Local Bifurcation Tilt:       119.037559509277
StdDev of Local Bifurcation Tilt:       16.0942306518555

Minimum   Local Bifurcation Torque:     0
Maximum   Local Bifurcation Torque:     180
Average   Local Bifurcation Torque:     79.3521270751953
StdDev of Local Bifurcation Torque:     51.1045875549316

Minimum   Ralls Power Constant:         0.2886962890625
Maximum   Ralls Power Constant:         3.4918212890625
Average   Ralls Power Constant:         0.870361328125
StdDev of Ralls Power Constant:         0.773306369781494

Minimum   Ralls Power Correction:       0.0542905852198601
Maximum   Ralls Power Correction:       50.2750625610352
Average   Ralls Power Correction:       3.51009440422058
StdDev of Ralls Power Correction:       10.2176713943481

Minimum   Remote Amplitude Angle:       10.6016492843628
Maximum   Remote Amplitude Angle:       168.301834106445
Average   Remote Amplitude Angle:       49.6291542053223
StdDev of Remote Amplitude Angle:       34.4641380310059

Minimum   Remote Bifurcation Tilt:      37.3422698974609
Maximum   Remote Bifurcation Tilt:      169.190765380859
Average   Remote Bifurcation Tilt:      139.069442749023
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Fig. 5: A draft presentation of a txt format file containing morphological parameters of a traced neuron, as it is automatically extracted by Neuromantic software

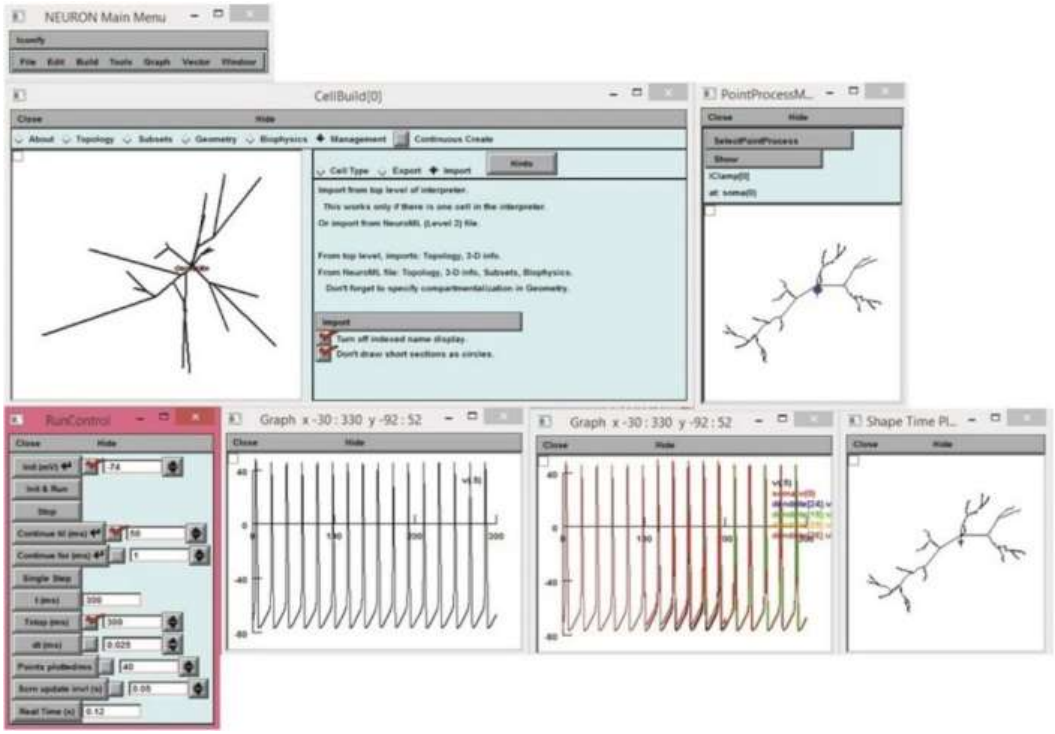

Fig. 6: NEURON Simulation environment is used to study the influence of the morphological parameters to the neuronal functionality

From the time the brain is removed until coverslipping, neurons undergo significant changes in morphological structure. To ensure that autolysis time does not affect dendritic measurements, two-tailed Pearson product correlations can be performed between all dependent measures and autolysis time and in the case that a significant correlation is noticed this has to be taken into account.

Other options such as confocal microscopy of neurons filled with fluorescent tracers could, in principle, be more accurate and might even contribute an element of automation to the reconstruction process.

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