Morphological Analysis of Individual Neurons

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Abstract. 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. Neurons are themselves complex computational machines. Theories of dendritic, somatic, and axonal functions have matured well beyond the traditional scheme of "input–integration–output". Single neurons and their arbors are now considered sophisticated time filters, coincidence detectors, internally distributed devices of local memory storage, and dynamic metabolic assemblies with high internal spatial specificity. The first step in the pathway of morphological analysis of individual neurons is the neuronal staining either with intracellular labeling techniques, or with the traditional silver staining methods. Another fundamental step is the tracing into a three-dimensional (3D) digital representation of the branching dendrites and/or axons, and the last and crucial step is the mathematical and statistical analysis of the acquired morphological parameters which can be classified in two main categories, the topological and geometric parameters.

Keywords: neurons, morphology, neuropsychiatry, memory

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 [1].

　　　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 and further on how the affecting on the CNS connectivity results in neuropsychiatric disorders and all the associated deficits that come along with that, as our groups previously showed in an extensive matter [2-9].

　　　The establishment of neuroanatomical databases and the development of computer graphics have resulted in a plethora of high-level research projects focusing

on computational modeling of neuroanatomy [10,11]. These studies range from the description of dendritic morphology and the characterization of its relationship with electrophysiology to the analysis of the structural determinants of higher brain functions via the detailed mechanism of neuronal assemblage into functional networks.

Neurons are themselves complex computational machines. Theories of dendritic, somatic, and axonal functions have matured well beyond the traditional scheme of "input–integration–output". Single neurons and their arbors are now considered sophisticated time filters, coincidence detectors, internally distributed devices of local memory storage, and dynamic metabolic assemblies with high internal spatial specificity [10,12].

Which neuronal substructures are the elementary computational units of the brain? For the time being, however, much still needs to be discovered about neuronal morphology, and computational studies have, so far, mainly considered dendrites as the "elementary" structural objects for modeling purposes [13].

Dendrites constitute the first step in the bottom-up path towards an integrated structural model of the brain. Although neurons can be classified according to a variety of criteria, including location within the nervous system, main neurotransmitters released, presence of specific protein markers, dendritic and axonal structure, and interconnectivity with neurons of other classes, dendritic morphology is traditionally a fundamental criterion in neuronal classification, partly because it is immediately captured by optical microscopy under staining conditions discovered and optimized over a century ago. Dendritic trees come in all shapes and sizes [13,14]. They range from a total length of a few tens of micrometers to a few millimeters. Some neurons have only one main dendritic branch, while others possess up to 15–20. Some branches meander strongly, while others are approximately straight. Dendritic morphologies vary significantly even within one neuronal class [15]. In addition to this morphological diversity, the molecular composition of ion channels in the membrane strongly differs along the stretch of one dendrite, and more pronounced differences even exist between neurons of different types [16].
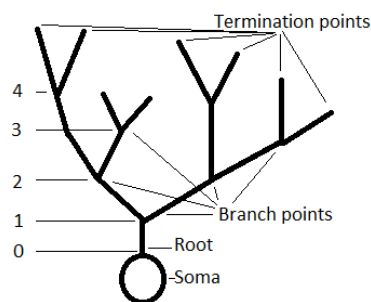

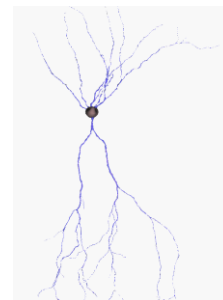**Figure 1:** Morphometric analysis of dendrite morphology

Figure 2: Representation of a fully traced pyramidal neuron

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Neuronal staining

In the last few decades, the development of intracellular labeling techniques using horseradish peroxidase (HRP) and biocytin, combined with computer-assisted methods for quantitative reconstruction of labeled neurons has led to a large output of high resolution data about dendritic morphology. Tracer injections are typically performed in single neurons in vivo or in vitro, and microscopy rendering often involves fixing and reslicing the tissue [17].

For about a century the Golgi technique has been very successful in staining neurons enabling the semi-automatic reconstruction and the quantitative analysis of their neuronal branching patterns. In combination with other classical staining methods it has been used to achieve a quantitative statistical description of brain tissue in terms of the density of neurons, synapses, and total length of axonal and dendritic arborizations. The Golgi methods comprise several different metallic impregnation techniques. These methods have in common, the property of impregnating cytoplasm with metallic salts, which render the entire profile of an individual cell and its processes visible in histological preparation suitable for light or electron microscopy [15,18].

Although Golgi methods have taken second place to other techniques involving intracellular labelling, yet there new methods never came close to matching the overview of entire brain areas that Golgi preparations can provide. The fact remains that even after 130 years of its discovery; the technique is increasingly used, not only in its primary role in qualitative histology, but as a keystone in the new quantitative neurobiology, experimental neurology, neuropathology and neuromorphology [18 19].

The main strength of the Golgi method lies in its capacity to reveal all components of the nervous system; neurons, glia and vascular system. Only a small percentage of the neurons in any one area (1-10%) are impregnated in a single preparation. Of the neurons rendered visible, virtually all portions can be seen. The cell body, dendrites, dendritic substructures (spines) and at least part of the axon are visible. It provides a panoramic view of the entire neural element in black on a yellow or pale orange background [20].

The basic method was initially discovered by Camillo Golgi (1873), which involved the exposure of brain tissue to dichromate followed by impregnation with heavy metal ions either silver or mercury. The original method of Golgi was very time consuming i.e., immersion of the tissue in potassium dichromate for several months and subsequent impregnation in dilute silver nitrate for many days [21].

The genius of Santiago Ramon Y Cajal was compounded by profound biological insight, skillful exploitation of existing techniques and invention of new ones, consummate artistry and an enormous capacity for sustained hard work. In 1887, he took up the neurohistological staining method, which, although introduced by Golgi, was modified and referred to as rapid Golgi staining procedure [1]. During the last century numerous modifications have been developed, with the most important of them being the following:

- Cox (1891) developed another modification called the Golgi-Cox method that is particularly useful for tracing dendritic arborization [22].
- Hortega del Rio (1928) used formalin with the dichromate salt and chloral hydrate and this variation of rapid Golgi method is particularly useful for studying the neuroglia, small granule cells and the cerebellum.
- Fox et al. (1951) described a variation of rapid Golgi method for use with formalin fixed brain tissue, which is useful foe adult tissue.
- Many other variations of the above mentioned methods have been described in the literature.

Neuronal tracing

The first and critical task in the study of neuronal morphology is the selection of neurons, which will be traced. 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.

A fundamental step for computational neuroanatomy is the tracing of the acquired neuronal images into a three-dimensional (3D) digital representation of the branching dendrites and/or axons. Traditionally performed manually, this process is very labor intensive severely limiting the number of available reconstructions. Both commercial (Neurolucida, MicroBrightField) and freeware software systems (Neuromantic – Figure 3, NeuroMorpho, or NeuronStudio) offer some level of automatization of the reconstruction process.
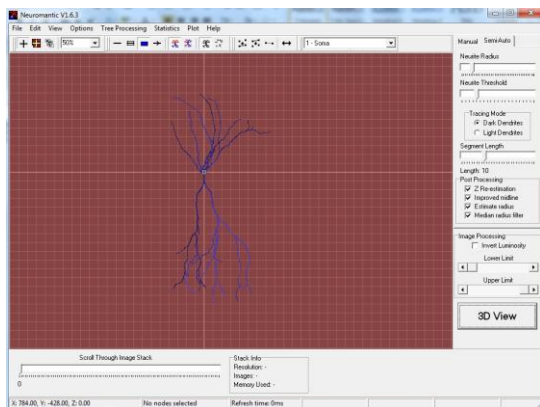

Figure 3: Neuromantic is one of the open source applications that can be used for neuronal tracing and statistical analysis

Taking into account the amount of required human intervention, four main classes can be distinguished:

1. Manual (Camera lucida). Prisms are employed to visually overlay the microscope image onto a piece of paper, and the neuron is then traced by hand. Although primarily used for 2D tracings, 3D reconstructions can be derived from these with time consuming post-processing [23].

2. Semi-manual (e.g., Neuron_Morpho, Neurolucida). Digital segments are added by hand through a software interface, typically sequentially, beginning at the soma, and working down the dendritic tree.

3. Semi-automatic [24]. User interaction defines the basic morphology, such as identifying the tree root and terminations, but branch paths are traced by the computer.

4. Fully automatic [25]. The entire morphology is extracted with minimal user-input.

The development of such techniques and increasing computational power and memory allow the collection of greater amounts of morphological data and execution of more complex analyses. The purpose of semi-automatic methods is to provide significant assistance in tracing neurites; rather than forcing the user to manually segment each point along a dendrite, clicking on two positions on a neurite will automatically trace along it [26].

Theoretically, fully automatic tracing should be able to produce a full and accurate 3D reconstruction of a neuron from an image stack with minimal user-input. Hence, in principle, fully automatic methods should be highly preferable to semi-manual tracing. In practice, however, most tracing is still performed semi-manually with applications such as Neurolucida [26]. The primary reason for this is inaccuracy: the time required to edit the results of an automatic reconstruction in order to obtain the desired accuracy is greater than the time required to perform a semi-manual reconstruction. Additionally, such algorithms tend to be restricted to high-quality imaging technologies such as confocal or electron microscopy. If dendrites can be distinguished from the background by luminosity alone via global thresholding, the morphological reconstruction may be achieved with a skeletonization algorithm. However, such imaging technologies are still less widely available in neuroscience laboratories than standard widefield microscopes, due to significantly higher cost [27].

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 [26, 27].

Morphometric Measurements

Morphometric measurements are divided into two main categories, the topological and geometric parameters. The topological morphometrics deal with the branching structure of the tree independently of metric units, for example number of branch points, number of dendrites and branch order, while the geometric parameters have metric or angular units. Another distinction between morphometric parameters is that between global/scalar, such as total length and local/vector features such as the individual lengths.

Topological parameters

The main topological parameters are the number of stems defined of edges leaving the dendritic root/soma, the number of branch points, and the number of termination points. The number of stems leaving the neuronal soma is usually used to classify cell types, while the number of branch and termination points represents the extent of branching in a tree. The number of termination points can be calculated by the equation **TP=BP+1**, where TP equals the termination points and BP the branch

points. The branch order is computed following the centrifugal order as follows: the dendritic root has by convention an order of zero, so that the order of a node becomes one plus the number of branch points encountered on the path between the inspected node and the dendritic root.

The term dendritic order is defined as the number of termination points in the sub-tree rooted at the node under investigation. The distribution of the order and degree in a dendritic tree can be used for classification and description of dendritic trees (Verwer and van Pelt 1983). Both dendritic order and degree can be used in combination with other morphometric features to create conditional distributions, such as dendritic branches per order, or dendritic length per dendritic order.

Another composite topological parameter is the partition asymmetry which refers to the topological complexity of a neuronal dendritic tree on the normalized difference between the degree of two daughter branches at a branch point, and ranges from 0 for completely symmetric to 1 for completely asymmetric trees [28,29]. An additional composite parameter which refers to the relation of asymmetry with the depth of the tree is the *Horton-Strahler (HS)* index.

The HS index is computed at each branch point and equals *k+1* when both daughter branch points have equals HS index of k or as max(*k1, k2*) when the *HS* indexes of its daughters k1 and k2 are not equal. The Strahler number is defined as the *Horton-Strahler index* associated with the root of the tree.

Geometric parameters

The main parameters in this category are the segment length values and diameters, including the stem length, the interbranch point length and the terminal segment length, but the most basic one is the total dendritic length. Relations between different locations in the tree can be described by a length metric in terms of Euclidean distance or the path length between those two points, where Euclidean distance is the "ordinary" straight-line distance between two points in Euclidean space, and path length refers to the full length following the path of the dendritic tree from one point to the other. The relation of the segment length to the Euclidean distance from the dendritic root can be expressed by the somatofugal tropism factor [30,31] which refers to the ratio of the segment path length and the Euclidean distance between the starting and end point, and is 1 for a segment growing radially away from the root/soma, or 0 for a segment that grows concentrically in relation to the root/soma.

The fractal dimension is a measure of self-similarity and is often used as a measurement of space-filling [32], and by definition a straight line has a dimension of 1, a square has 2, and a cube has 3. The interpretation of the fractal dimension is arbitrary and depends on the method used to calculate it. In the calliper method [33], the fractal dimension represents the level of meandering of a dendrite, where a straight line has a dimension of 1 and more meandering dendrites receive slightly higher values. Because the validity of the fractal dimension is disputed in the analysis of neuronal morphologies [34, 35], the dendritic contraction can be used as a proxy of the fractal dimension. Contraction

is defined for a stretch of dendrite between those points as the ratio of the Euclidean distance and the associated path length between those points. A straight line has a contraction of 1, while meandering dendrite has a slightly lower value. Intuitively, the relation between contraction and the fractal dimension can be approximated as fractal dimension for planar dendrites. Both contraction and fractal dimension quantify space-filling. The relation of the change of dendritic diameter along the neuronal processes can be approximated by the tapering rate which represents the linear or the nonlinear rate at which the diameter decreases per unit of length, while discontinuities in the tapering rate occur at branch points and can be referred to by the child-parent ratio, the ration between the diameters of the parent and the child segments.

Sholl analysis

Sholl analysis is a method of quantitative analysis of dendritic arbors, first described by Sholl in 1953 [36]. While methods for estimating number of cells have vastly improved since 1953 with the advent of unbiased stereology, the Sholl analysis is still in use. This method can be performed on both two and three dimensional neuronal representations and gives an objective representation of the dendritic tree density and complexity. Using the Sholl analysis, a mathematical algorithm named the branching index (BI) has been described to analyze neuronal morphology. The BI compares the difference in the number of intersections made in pairs of consecutive circles of the Sholl analysis relative to the distance from the neuronal soma. The BI distinguishes neurons with different types of neurite ramification [37].

Common methods include Linear Analysis, Semi-log Analysis and Log-Log Analysis.

Linear Method

The Linear Method is the analysis of the function N(r), where N is the number of crossings for a circle of radius r. This direct analysis of the neuron count allows the easy computation of the critical value, the dendrite maximum, and the Schoenen Ramification Index [*37*].

Critical Value: The critical value is the radius r at which there is a maximum number of dendritic crossings, this value is closely related to the dendrite maximum.

Dendrite Maximum: This value is the maximum of the function N(r), as specified by the Critical Value for a given data set.

Schoenen Ramification Index: This index is one measure of the branching of the neuronal cell being studied. It is calculated by dividing the Dendrite Maximum by the number of primary dendrites, that is, the number of dendrites originating at the cell's soma.
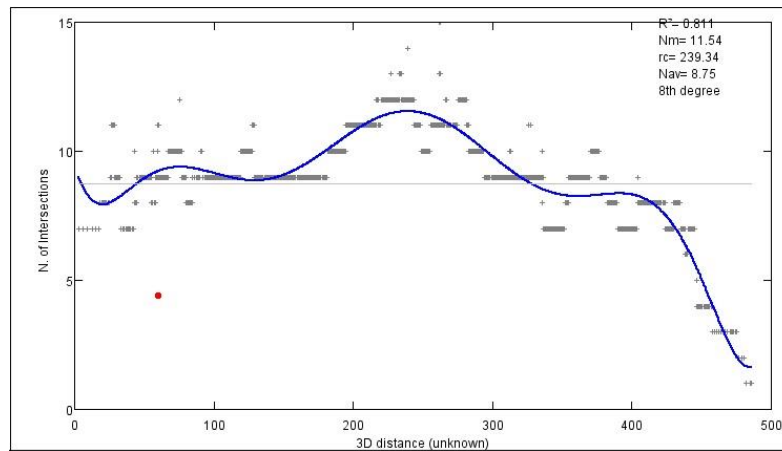
Figure 4: Graphic representation of three dimensional Linear Sholl analysis

Semi-Log Method

The Semi-Log Method initiates by calculating the function Y(r) = N/S where N is the number of dendrite crossings for a circle of radius r, and S is the area of that same circle. The base 10 logarithm is taken of this function and a first order linear regression, linear fit, is performed on the resulting data set, that is:

$$\log_{10}\left(\frac{N}{S}\right) = -k \cdot r + m$$

where k is Sholl's Regression Coefficient [36].

Sholl's Regression Coefficient is the measure of the change in density of dendrites as a function of distance from the cell body [38]. This method has been shown to have good discrimination value between various neuron types, and even similar types in different regions of the body.

Log-Log Method

Closely related to the Semi-Log Method, the Log-Log Method plots the data with the radius plotted in log space. That is the researcher would calculate the value k and m for the relation:

$$\log_{10}\left(\frac{N}{S}\right) = -k \cdot \log_{10}(r) + m$$

This method is used in a manner similar to the Semi-Log Method, but primarily to treat neurons with long dendrites that do not branch much along their length [38].

Modified Sholl Method

The Modified Sholl Method is the calculation of a polynomial fit of the N and r pairs from the Linear Method.[6] That is, it attempts to calculate a polynomial such that:

$$N(r) = a_0 + a_1 * r + a_2 * r^2 + \ldots + a_t * r^t.$$

where t is the order of the polynomial fit to the data. The data must be fit to each of these polynomials individually, and the correlation calculated in order to determine the best fit. The maximum value of the polynomial is calculated and used in place of the Dendrite Maximum. Additionally, the average of the resulting polynomial can be determined by taking its integral for all positive values represented in the data set.
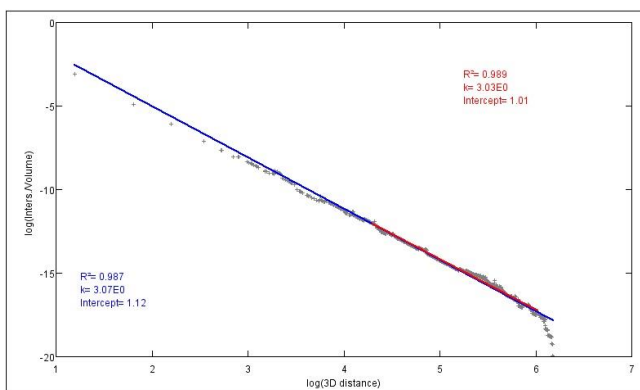

Figure 5: Graphic representation of three dimensional logarithmic Sholl analysis

Similarity

The morphometric measures can be used as a metric to quantify similarity between neuronal tree morphologies and rank them on their similarity using standard statistical tests such as Student's test for vector parameters, or Kolmogorov-Smirnov test as a nonparametric alternative. Comparisons can be done between univariate or multivariate parameters and in addition to that there are other dedicated measurements to quantify similarity with the tree distance which formalizes how many operations have to be made to morph one tree into another [39] being one of them, and the shape diffusion index which is technically more complex and can be used as a measure of how easily a morphology can be synthesized [40].

Limitations

It has become clear in recent years that neuronal dendrites are not static structures; rather they can exhibit dynamic changes that presumably reflect functional changes in the nervous system. Thus, the data about dendritic structure that are obtained by conventional neuroanatomical methods represent snapshots that may not be entirely representative. Second, there are a large number of practical difficulties inherent in gathering quantitative measurements of neuronal dendrites using conventional light microscopy. These include factors such as tissue shrinkage, operator error, and the limited resolution of the light microscope, which is the only practical approach to reconstructing large neurons from serial sections. These sources of potential error must be kept firmly in mind when evaluating existing data, particularly when data from different sources are combined. From the time the brain is removed until coverslipping, neurons may undergo significant changes in

morphological structure. Even just the event of slicing a brain and subsequent incubation may change the morphology significantly by triggering the growth of a sizable proportion of dendritic spines within hours. Thus, it may be difficult to obtain cell morphologies that fully preserve the in vivo situation from slice tissue. A comparison with cells of the same type obtained from perfused brains can help judging the presence of slice artefacts such as swelling or spine growth. Both in slices and in perfused brains, neuron morphologies will get distorted to a varying degree during fixation and subsequent histological processing. Before relying on the obtained morphology, some cross-validation of this process is advisable. Of course, the need for such a validation depends on the accuracy that the modeler desires to achieve with morphological reconstructions [41].

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 center, leading to a distortion of cells. Also, individual dendrites may curl up rather than shrink, and curled dendrites may retain their original length [41].

Light microscopy (LM) is limited by the optical resolution that can be obtained. The fundamental limit is approximately 0.6 * / N.A. where is the wavelength of the light and N.A. is the numerical aperture of the objective used. For a typical wavelength of 500 nm and a numerical aperture of 1.0 the limit in resolution is thus 0.3 m. Numerical apertures of up to 1.5 can be achieved with oil-immersion objectives, but the working distances of high N.A. lenses are relatively short, and generally don't allow focusing deep enough to visualize stained cells in thick brain slices. A resolution limit of 0.3 m does not mean that thinner structures are not imaged. Rather, a thin line of 0.1 m structure will have an apparent diameter of about 0.3 m due to light diffraction. This effect leads to an apparent increase in diameter of small dendrites that can lead to a significant error in estimates of axial resistance and membrane surface area. When cells with abundant small profiles are reconstructed for the purpose of accurate passive modelling, a calibration of processes with small diameters using EM is therefore recommended. This is particularly relevant when accurate reconstructions of axon collaterals are desired [41].

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. However, technical problems, such as tracer bleaching, have confined most reconstruction efforts to more permanent forms of tracers, like HRP or biocytin, to be examined with conventional light microscopy. With regard to data analysis, it is also important to remember that the process of quantitative reconstruction splits the continuous structure of the dendrite into discrete pieces, here referred to as "segments", each with a specified diameter and length.

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