Detection and Identification of Neurons in Images of Microscopic Brain Sections

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Abstract. This paper presents a new combined mathematical method, which were proposed, implemented, and experimentally tested for extracting information necessary for modeling and, in future, predicting Parkinson's disease. The method was developed for extraction "neurons" from microscopic images of brain slices of experimental animals. Then it was adapted for different types of initial data, because unfortunately the quality of initial images depends on skills of the specialist who has done an experiment. Now the method allows one to detect and identify as neurons a set of small informative extended objects with well distinguished (by brightness) oval inclusions. The result is a binary image of the contours of detected objects and their inclusions and a list of characteristics calculated for each detected object. The method is based on the joint application of image processing methods, methods of mathematical morphology, methods of segmentation, and the methods of classification of microscopic images. The method was applied to the following areas of brain: the substantia nigra pars compacta and the arcuate nucleus of hypothalamus.

1 Introduction

One of the most important problems of neurosciences is the development of experimental models of socially important neurodegenerative diseases, in particular, those associated with the death of dopaminergic (DAergic) neurons. The degeneration of the latter in the human nigrostriatal system leads to the development of Parkinson's disease (PD) [2]. These models are designed for the development of new methods and technologies for the diagnosis and treatment of such diseases. The models could be developed much faster and would be economically more effective, with reduced time and material expenses for morphological studies. The latter can be reached by the automation and optimization of the methods for processing and analysis of experimental data. In particular, the automation of the analysis of images of neurons and their dendrons obtained from the microscope (MINs) makes it possible to reduce material costs by an order and the time costs, by two orders [7].

In spite of the fact that interest to the problem of analysis of biomedical images, including MINs, is being constantly growing, there have been very few successful attempts to automatize the process (or its stages) (see, for example, [3, 9, 14]). The detection and tracing of neurons in two dimensional microscopic images of brain

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slices is complicated by the following factors [9]: (a) MINs contain, in addition to neurons, objects that are not neurons (particles of dirt, staining errors, tissue folds, blood vessels, and other artifacts); (b) neurons in MINs may strongly differ from each other both in size and shape; (c) neurons may be damaged during the preparation of slices; this fact affects their shape and, hence, leads to a large number of cells of different types on a slice; and (d) neurons may stick together or may be overlapped. On the whole, one should admit that the concept of a neuron as a visual object is not clearly defined, and there is no universal list of contextual, logical, geometrical, qualitative, and quantitative conditions and characteristics that would allow one to standardize the description/definition of this visual object.

As a rule, the detection and tracing of a neuron in histological microscopic images involves the following main stages: preprocessing, segmentation, and classification. The need for the preprocessing of a MIN is attributed to the presence of noise, low resolution of a MIN, and the contrast non-uniformity due to error in staining the slices. To improve the quality of MINs, one usually applies standard operations of image processing such as smoothing, inverse convolution, morphological filtration, and some other operations [12].

In the publications devoted to the automatic or semiautomatic detection of neurons in MINs, one mainly uses, for segmentation, algorithms based on thresholding (for example, [3, 10]), morphological operations (for example, [4]), Potts models (for example, [13]), and watershed methods (for example, [15]) or active contour models (for example, [5, 9]). The further classification is carried out, for example, with the use of Bayesian procedures, principal component analysis, or machine learning methods (for example, [1]).

An important stage in the analysis of the MINs of brain slices is the morphological characterization of the detected neurons. In recent years, a wide set of specific morphological parameters has been defined for the efficient mathematical characterization of the morphology of neurons, including their nuclei and dendrons (see, for example, [11]).

A survey of the methods and systems of analysis and recognition of MINs for solving the problems of automation of diagnosis and the prediction of the clinical course of neurodegenerative diseases has been published by the authors of the present paper in [6].

The mathematical apparatus developed by the authors is based on the combined application of the methods of mathematical theory of image analysis and mathematical theory of pattern recognition, mathematical morphology, descriptive image algebras, information theory, and the methods of mathematical statistics. The methods developed allow one to efficiently detect and extract informative objects in microscopic images of brain slices (under restrictions imposed on the shape, size, topology, and the smoothness characteristics of the boundaries of objects [7, 8]).

2 Statement of the Problem

The problem consists in detecting the contours and calculating the morphofunctional characteristics of neurons for constructing a PD model that represents differences

between the parameters of DAergic neurons in the test and control groups (experimental animals from the test group were subjected to neurotoxin).

As a source of experimental data for constructing a PD model, we used digital microscopic images of DAergic neurons and the fibers of brain slices of experimental animals (with a resolution of 0.0117μm2/pixel2).

First of all, DAergic neurons are found on frontal serial slices of the substantia nigra pars compacta (SNC) with a thickness of 20 μm (fig. 1, left figure). They represent a key link in the regulation of motoric behavior. A progressive degeneration of these neurons leads to the development of PD.

DAergic neurons represent dark oval cells with light nucleus. The shape of DAergic neurons may be rather arbitrary; however, in many cases one can observe a convex soma surrounding a nucleus. The mean diameter of DAergic neurons ranges from 10 to 20 μm. The nuclei are of oval shape, with a minimum diameter of 6–12 μm and a maximum diameter of 9–15 μm. The relative size of the body (soma) and the nucleus vary strongly (one may observe either a very thin soma around the nucleus or a relatively small nucleus compared with the area of the soma). MINs contain a large number of fibers around a cell, which may mask the cell, forming an intense background around a neuron.

In addition to the factors, listed in the Introduction, that impede the processing of MINs, one should take into account that (a) MINs contain regions of brain with a different number of neurons per unit area, and (b) microscopic images of different brain slices differ strongly in contrast and brightness.

DAergic neurons are found on frontal serial slices of the arcuate nucleus of hypothalamus (AN) with a thickness of 20 μm (fig. 1, right figure). The DAergic neurons in this part of brain are the same as in SNC area, but the initial images of slices have significant differences: 1) the background of AN images is brighter than the background of SNC images; 2) neurons located in AN slices can have no visible cores; 3) average number of AN neurons per slice is in several times smaller than there are in SNC area; 4) neurons located in AN slices are distributed rarely than in SNC slices and mutual contacts between neurons in AN area are practically excluded.

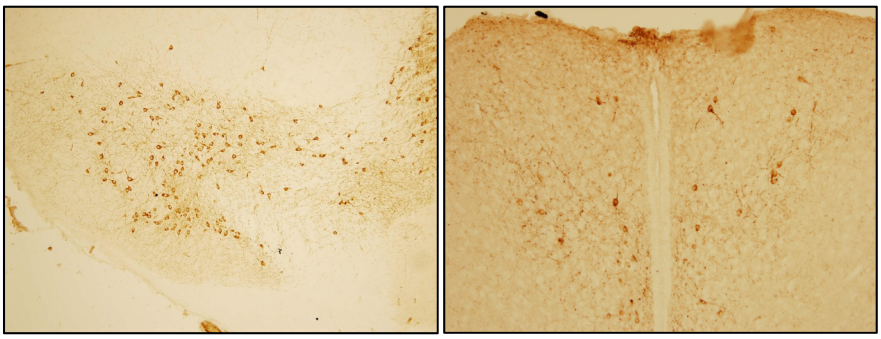

Fig. 1. Examples of images of neurons and their dendrons obtained from the microscope: a) SNC area (left figure); b) AN area (right figure).

3 Automated Detection and Identification of Neurons in Microscopic Images of Stained Brain Slices

The theoretical basis of the development of a new combined method for the analysis of MINs is the descriptive approach to image analysis and understanding [8]. According to this approach, a mathematical method for the analysis of MINs is represented as a special algorithmic scheme.

The method is designed for detecting a set of small informative extended objects with well-distinguished (by brightness) oval inclusions in microscopic images of frontal slices of the SNC and AN and calculating their morphometric characteristics.

The result is a binary image of the contours of selected objects and a list of characteristics calculated for each detected object. The method is based on the combined application of image processing methods, methods of mathematical morphology, methods of segmentation, and methods of classification of microscopic images.

The main feature of the method is that, after preprocessing of images, an iterative analysis of microscopic images is carried out with a view to distinguishing various classes of objects, that involves five procedures: (1) objects different from neurons are eliminated at the stage of application of the classifier; (2) a special class of objects-stuck-together neurons- is distinguished after the classification; the analysis of these neurons reduces to the construction of boundaries between two neurons; (3) segmentation ensures the separation of "good" neurons (neurons with regular shape); (4) analysis of neurons with thin soma whose boundary has been partially erased and the nucleus is in contact with the background; and (5) elimination of the dendrons of neurons.

The combined method involves the following main steps: (1) preprocessing of MINs: (1.1) filtration of MINs by an unsharp mask, (1.2) normalization of MINs, (1.3) morphological closing by reconstruction, and (1.4) binarization of MINs; (2) analysis of MINs: (2.1) segmentation of MINs, (2.2) classification of detected objects, (2.3) tracing neurons, (2.4) tracing the boundaries of neurons, (2.5) cutting off the dendrons of the neurons, and (2.6) construction of the boundaries of detected neurons; and (3) morphological characterization of neurons.

The algorithmic scheme for analysis of AN slices is almost the same: 1) the step 1.4 of MINs binarization is applied with other values of parameters; 2) the step 2.2 of object classification is based on another method of learning classifier.

The algorithmic scheme that implements the detection and identification of objects involves the following stages:

1. Preprocessing of MINs:

1.1) Filtration of MINs by an unsharp mask: the goal of this procedure is to increase the sharpness of the MINs, which allows one to increase the accuracy of tracing closely located neurons at further stages; the input data is a color MIN, and the output data is a sharper color MIN.

A copy of the initial image is subjected to blurring (standard Gaussian blurring). If the difference between the mask and the original exceeds a certain threshold, the images are subtracted. The threshold is needed to avoid undesirable details such as noise in a digital image. The method ends with a pixel-by-pixel addition of the initial and obtained images.

Unsharp masking increases the local contrast of an image in the regions that initially contained sharp variations in color gradation; this allows one to keep the boundaries between neurons.

1.2) Normalization: the aim of this procedure is a transition to a grayscale representation of MINs to normalize the difference in the staining of preparations; the input data are a color MIN with increased sharpness, and the output data is a grayscale MIN.

The formula for the transition from a color to a grayscale MIN is standard, because all three channels of the original image carry equal information necessary for the further operation of the method. To pass from a color to a grayscale image, one should apply the following formula for calculating the gray level at every point of this image: gray= 0.299*R+ 0.587*G+0.114*B.

1.3) Filtration of MINs with the use of morphological closing by reconstruction; the aim of this procedure is to suppress dark noise objects that are smaller than neurons; the input data is a color MIN, and the output data is a grayscale MIN.

The applying of a morphological filter is motivated by the fact that the initial MINs contain noisy objects that neither belong to the objects of the background nor represent the goal objects. Noise in the images is produced due to the parts of neurons (a part of the neuron shell) that do not completely fall into the slice, or due to the terminals that fall into the slice. The noise objects in the image distort the results of classification; moreover, they may affect the shape of the selected objects in case of superimposition.

For the morphological processing of images, we chose a filter “closing by reconstruction” because of the applying of this filter preserves the boundaries of the objects subjected to filtration.

After the morphological filtration, large objects of the foreground that can easily be detected against the background remain on the grayscale image. However, large noisy objects may also exist among the remaining objects. This problem is solved at the subsequent steps of the method.

1.4) Binarization; the aim of this procedure is a transition from a grayscale MIN to a binary MIN followed by the segmentation of the objects; the input data is the processed grayscale MIN, and the output data is the binary MIN with the goal objects, which will be checked for membership in the “neuron” class.

The binarization of an image is performed by a thresholding algorithm with adaptive threshold. The adaptive threshold is chosen by Otsu’s method [12]. After that a threshold rule is applied to the grayscale image: all pixels of the original image whose brightness function is above the threshold are assigned a brightness value of 255, and the remaining pixels are assigned a value of 0.

The binarization of AN images is performed by a thresholding algorithm with adaptive threshold multiplied by some normalizing constant, because of the brightness of the background. The constant (0.74) was found during the experiments.

The pixels with a brightness value of 0 on the binary image obtained are considered to be neuron like objects, while the pixels with brightness 255 are assumed to belong to the background.

2. Analysis of MINs:

2.1.2) Elimination of objects according to the shape and size of neurons; the aim of this procedure is to eliminate redundant objects by their size (a) from above with a very high threshold, in order to remove large areas that are certainly not neurons,

while retaining fragments corresponding to stuck together neurons, and (b) from below with a small lower threshold, in order to remove undoubted noise; the input data are the extracted connected components, and the output data is a shortened list of connected components.

2.1.3) Preparation of areas with extracted objects for classification; the aim of this procedure is the construction of the minimal bounding square for each connected component; the input data is a binary MIN and the list of connected components, and the output data is the list of minimal bounding squares.

2.2) Classification of extracted objects; the aim of this procedure is the distribution of detected objects into two classes ("neurons" and "other objects"); the input data is the list of squares that contain connected components, and the output data are two lists of squares (the class of "neurons" and the class of "other objects").

The problem of recognition of neurons is solved by a classifier based on the calculation of the distances from some special points of objects (the search of these points is done during classifier teaching process) to the objects' edge in fixed direction and on the features based on such distances.

The classifier is applied with various resolutions to square fragments of a MIN that contain connected region chosen at the previous step. This approach allows one to determine the presence of a single or several neurons in a single connected region.

The accuracy of the trained classifier on a test sample was 5% (for AN - 7%) - error of the first type (neurons assigned to the class of "other objects") or 15% (for AN - 20%) - error of the second type ("non-neurons" identified as "neurons"). The accuracy of the classifier can be improved by increasing the learning sample and by bootstrap estimates to choose background objects.

2.3) Tracing "neurons":

2.3.1) Elimination of "other objects"; the aim of this procedure is to remove connected components corresponding to "other objects" according to the classification performed; the input data are the minimum size squares bounding "other objects" and the processed connected components, and the output data is a shortened list of connected components.

All the connected components are eliminated according to the list of minimum size squares bounding "other objects".

2.3.2) Checking the number of extracted "neurons" in a connected component; the aim of this procedure is to calculate the number of neurons in one connected component; the input data are the minimum size squares bounding neurons and the shortened list of connected components, and the output data is the number of extracted neurons in each connected component.

2.4) Tracing the boundaries of neurons:

2.4.1) Splitting stuck-together neurons; the aim of this procedure is the construction of a boundary between stuck-together neurons; the input data is the number of extracted neurons in each connected component and the binary MIN, and the output data is a new list of connected components.

When a connected component contains several neurons, stuck-together neurons are separated. The separation was proceed as follows. One "non-neuron" connected component suitable in size is taken. The amount of "white color" regions completely covering by connected component is calculated in the next step. If the amount of such regions is more than one than the median perpendiculars for line connected its centers

conducted between these regions. These perpendiculars will be the part of new neurons boundaries.

After the boundary of new candidate for “neuron” is highlighted the minimum size square bounding it and the classifier is running.

2.4.2) The construction of the minimum convex hull around a connected component; the aim of this procedure is to construct the minimum convex hull, which allows one to detect both “good” neurons and neurons with thin soma and vanishes after preprocessing of a MIN; the input data is the shortened list of connected components, and the output data are the minimum convex hulls of the connected components.

2.4.3) Obtaining a traced boundary of a connected region; the aim of this procedure is to obtain the boundary of neurons; the input data is a new list of connected components, and the output data are the preliminary boundaries of neurons.

2.4.4) Checking the coincidence of the exact boundary and the minimal convex hull; the aim of this procedure is to obtain the exact boundaries of neurons: (2.4.4.1) when a region of the convex hull differs substantially from the traced boundary of a connected component, the region of the convex hull is taken as the boundary of a neuron; (2.4.4.2) when the region of the convex hull and the traced boundary either coincide or differ insignificantly, the boundary of the connected component is taken as the boundary of a neuron; the input data are the preliminary boundaries of neurons and the minimal convex hulls, and the output data are the exact boundaries of neurons.

2.5) Cutting off the dendrons of neurons; the aim of this procedure is to eliminate redundant parts of objects identified as “neurons”; the input data are the exact boundaries of neurons, and the output data are the exact boundaries of neurons without dendrons.

2.6) Construction of the boundaries of the nuclei of extracted neurons; the aim of this procedure is to remove the pixels belonging to the connected region from the domain enclosed inside the boundary; the remaining pixels belong to the nucleus by definition; the input data are new exact boundaries of neurons and a new list of connected components, and the output data are the exact boundaries of nuclei.

3. Morphological characterization of neurons.

3.1) Determination of the necessary feature space; the aim of this procedure is to determine a parametric model for the characterization of neurons. Biological experts consider the mean brightness, perimeter, area, form factor, optical density, and amount as the main parameters of the model.

3.2) The characteristics are calculated for individual neurons and their averaged values by slices and series of slices; the input data is a binary MIN with the boundaries of objects and the original colored MIN, and the output data are the values of features and parametric models of neurons.

4 Experimental Testing of the Method Developed

The method of analysis of MINs developed is convergent, stable with respect to small variations in the initial data, and has quadratic computational complexity. The method is software implemented and is used for the automation, filling, and analysis of

preclinical models of PD by experimental data at the Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences (Moscow, Russian Federation).

Experimental testing was carried out on 58 brain slices of the same animal. The accuracy of the results was estimated by verifying whether the distributions of neuron characteristics detected automatically coincide with those detected manually by the Kolmogorov–Smirnov criterion (the zero hypothesis).

The accuracy of the results was also estimated by comparing the mean multifunctional characteristics obtained under the automatic and manual tracing of neurons on randomly chosen brain slices (we took five brain slices). The averaged values of the characteristics of objects traced in the automatic and manual modes are shown in Table 1 (for SNC case) and in Table 2 (for AN case). These tables present also the correlation between the characteristics calculated in the manual and automatic modes. The results confirm that the automatic tracing of neurons is close to the manual tracing: the difference in the brightness and the area of the selected region is within admissible limits, while the difference in the perimeters and the shape factors of objects is attributed to the fact that it is very difficult to draw the exact boundary manually.

During the experimental investigations, we confirmed the following characteristics of the algorithms developed. 1. The algorithms guarantee the analysis and recognition of the images of neurons on two dimensional brain slices of experimental animals. 2. According to the statistical estimate of the Kolmogorov–Smirnov criterion, the accuracy of the automatic analysis of neuron images is comparable with the accuracy of visual analysis of neuron images, which is carried out when studying PD without computer-aided analysis of images.

Table 1. Comparison of the characteristics of objects detected automatically and manually in SNC.

Manual detection				
Image	Mean brightness (0...255)	Area (mm^2)	Perimeter (mm)	Shape factor
1	149,7	2,355	5,09	-0,06
2	145,9	3,046	6,08	-0,02
3	149,8	2,815	5,77	-0,03
4	146,4	2,756	5,31	-0,02
5	150,0	2,493	5,23	-0,02
Automatic detection				
1	149,1	2,403	5,42	-0,05
2	145,2	3,166	6,69	-0,03
3	148,1	2,848	5,66	-0,03
4	146,6	2,813	5,11	-0,04
5	148,4	2,508	5,37	-0,03
Correlation between the manual and automatic modes				
	0,95	0,99	0,87	0,78

Table 2. Comparison of the characteristics of objects detected automatically and manually in AN.

Manual detection				
Image	Mean brightness (0...255)	Area (mm^2)	Perimeter (mm)	Shape factor
1	122,1	2,239	4,79	-0,06
2	112,7	3,691	4,32	-0,03
3	117,0	2,512	6,15	-0,03
4	123,4	3,254	4,67	-0,02
5	122,7	2,417	4,30	-0,04
Automatic detection				
1	119,7	2,432	4,89	-0,05
2	111,0	4,843	5,13	-0,03
3	118,7	2,712	6,15	-0,02
4	123,1	3,373	4,47	-0,04
5	121,1	2,313	4,51	-0,04
Correlation between the manual and automatic modes				
	0,93	0,95	0,86	0,75

5 Conclusions

We have developed a new mathematical method and a software designed for extracting and characterizing DAergic neurons in microscopic images of brain slices in the SNC and in AN. The problem of detecting neurons in the images of SNC and AN slices has been posed by scientists from the Laboratory of Hormonal Regulations at the Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences. They also supplied images for processing and analysis. The experimental testing of the method has been carried out jointly. At present, we study the possibility of improving the mathematical and functional characteristics of the method by using combinatorial recognition algorithms that admit the input of spatial information and essentially employ contextual and logical conditions and constraints.

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