Automatic detection of neurons, astrocytes, and layers for NISSL-stained mouse cortex

Svetlana Nosova

1st author's affiliation
1st line of address
2nd line of address
Country (ZIP) code, City, State

nosova.sv.a@gmail.com

Ludmila Snopova

2nd author's affiliation
1st line of address
2nd line of address
Country (ZIP) code, City, State

lsnopova@nizhgma.ru

Vadim Turlapov

3rd author's affiliation
1st line of address
2nd line of address
Country (ZIP) code, City, State

vadim.turlapov@gmail.com

ABSTRACT

We present an image processing algorithm for automatic detection of cortex layers and cells from optical microscopy images for Nissl-stained mouse brain. For every layer of cortex we automatically detect a shape and localization of following cortex cells type: neurons of molecular layer, pyramidal neurons, stellate neurons, and astrocytes. The algorithm includes the steps of: preprocessing, neurons and astrocytes localization, neurons classification, refined cortex layer detection, neurons reclassification. For preprocessing we use converting to gray image, Gaussian blurring, converting to black-white image after background removing, and rough estimate of layers. We use morphological operations with variation radius of structure element for neurons localization and neurons classification.

Keywords

image processing; automatic detection; optical microscopy; NISSL-staining; mouse cortex; cortex layers; neuron; astrocyte; preprocessing; morphology features

1. INTRODUCTION AND RECENT SOLUTIONS

The problem of fully automatic segmentation, decomposition and analysis of microscopy data of mouse brain (and different part of it) is known in global community. A reconstruction of microscopy data is a first step for understanding, analysis, and simulation of brain. In the optical microscopy it is needed to create special algorithms and software for each type of staining, which would help human to analyze and to segment data, and, in particular, to give information for diagnosis. In this paper we focused on NISSL-stained mouse cortex because Nissl staining is the easiest and mass for optical microscopy mouse brain.

In [Kol14] researches described three types of segmented cells: neurons, glial cells, epithelial cells. Authors review necessary features for every type of the cells. They focused on segmentation of pyramidal neurons based on special preprocessing and watershed algorithm. But the paper has no

information about statistics of cortex layer and about quality of neurons and astrocytes in every layer.

In the community publications we can find a sufficient amount of sources about segmentation of neurons in NISSL-stained data. In [Das15] a segmentation based on machine learning (random forest) is used. [Hey15] describes algorithm based on normalized graph cut. Special method ANRA proposed in [Ing08]. It's also based on machine learning techniques and has good accuracy.

What about layer decomposition, we should notice that there are different 3D mouse brain atlases with information about brain areas and layers ([All04] and [Bra05]). And in [Sen11] we can see atlas-based segmentation of NISSL-stained mouse brain data. In [Mes15] we can find segmentation of hippocampus, and in [Bas16] you can see MRI-based segmentation of cortical area.

We analyzed NISSL-stained mouse brain data. The data for our research were provided by Nizhny Novgorod State Medical Academy. In this work we consider the follow questions: features and structure of different types of neurons and astrocytes; neuron localization detection; detection of layer localization and collection of statistics for neurons in every layer.

2. DATA DESCRIPTION

Input data is section set of mouse cortex images (see Fig.1). The input data is images set of the mouse cortex slice. There are intersections, i.e., lower

portion of the first image may include the same data as the upper part of the second image. It's problem for statistics collection and data processing.
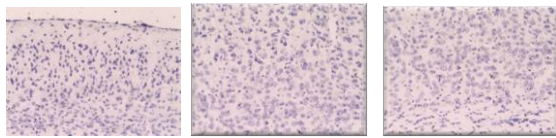

Figure 1: Section set of NISSL-stained mouse cortex.

We need algorithm and software for forming data in one image without duplicate parts. We implemented an automatic algorithm based on the algorithm for gluing panoramas as part of this work. But the algorithm was unstable, and because it was not the original purpose of this work, we decided to glue images by hand (see Fig. 2). This picture was marked by a professional pathologist.

Cortex layers, neurons and astrocytes feautures

Following cells are present on the images: molecular layer neurons (MLN), pyramidal neurons(PN), stellate neurons (SN), astrocytes. Also we have following layers in mouse cortex structure: layer I, layer II-III, layer IV, layer V, layer VI. In the layer I (the molecular layer) molecular layer neurons and astrocytes are presented. In the layer II stellate, pyramidal neurons and astrocytes are presented. In the layer IV only stellate neurons and astrocytes are presented. In the layer V all cells type are presented.and In the layer VI we can see only stellate neurons and astrocytes.
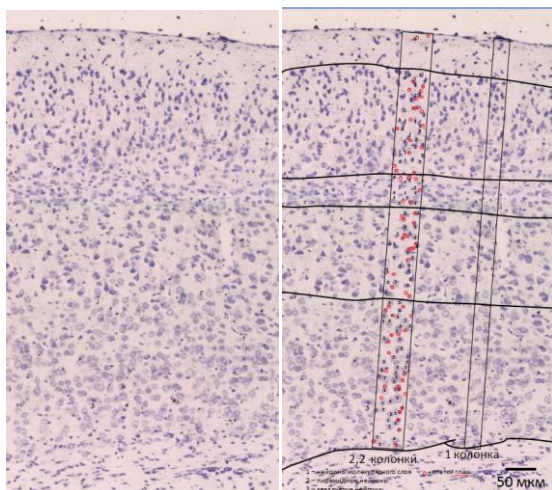

Figure 2: Thin slice (7 micron) of the mouse brain cortex in the original form, and marked up by a qualified morphologist.

We did hand segmentation to get astrocytes color statistics. For every type of neurons we did watershed segmentation from seed points and collect information about shape and color for every neuron

type. You can see summary color statistics information in Fig.3.

As you see, some part of neurons can be classified using only histogram information (as have different mean and dispersion for the intensity distribution). Shape characterization of every neuron type you can see in Tab.1. This information used for neuron classification.

We noticed also that pixel has color vector value less than $(200_r, 80_g, 200_b)$ for astrocytes.

	MLN	PN	SN
Area	448	965	1205
Perimeter	100	145	175
Extent	0,669	0,660	0,664
Minor Axis	19	30	33
Major Axis	34	47	50
Orientation	94	87	88
Aspect Ratio	0,595	0,645	0,669
Circularity	0,604	0,565	0,478
Solidity	0,862	0,858	0,813
Convexity	0,909	0,887	0,819
Equivalent Diameter	23	34	37

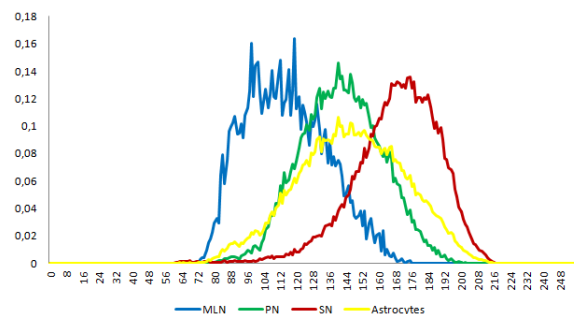Table 1. Shape features for different types of neurons.

Figure 3: Normalized intensity histograms for different cells type.

3. PROPOSED SOLUTION

We propose the algorithm for automatically layer detection, neurons type classification and astrocytes detection. Main steps of the algorithm are presented in Fig.4. Auto detecting of the layers and detailed statistics collecting are main goals for our work. You'll find detailed description for every algorithm step.

Preprocessing

Preprocessing is preparatory step for detection. It include two phases: noise removing and background removing. We use 5x5 Gauss filter for noise removing from source image. All next steps of

preprocessing are used for background removing. We form image in which colored pixels comply cells and black pixels are background. Detailed description of background removing with our comments you can find in Alg.1.
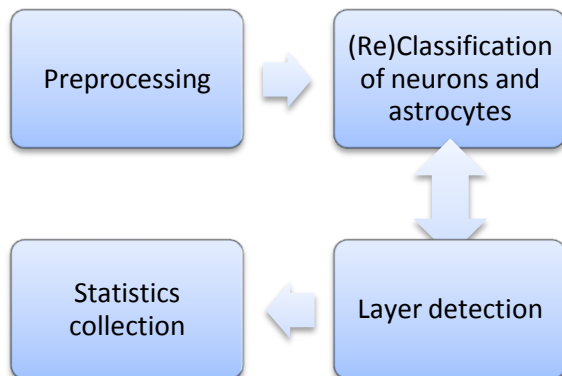

Figure 4: Algorithm for layer detection and cells classification.

If(source(i,j) >= min_background_value &&
source(i,j) <= max_background_value)

        bw(i,g) = white;

Else

        bw(i,j) = black;

Figure 5: First binarization pseudocode.

Step 1. Create a binary image *bw(i,j)* using algorithm in Fig 5. Parameters *min_background_value* and *max_background_value* are setted by human.

Step 2. Delete noisy pixels from image and create fill "black regions" on our image. Apply morphological erosion and get *erode_bw(i,j)* image.

Step 3. Invert *erode_bw(i,j)* and get *inv_bw(i,j)* .

Step 4. Find all contours on *inv_bw(i,j)* and delete all contour which less than *min_contour_area*. You need fill this contours using black color on *inv_bw(i,j)*. On this stage we can see some too big white regions, in the center of which placed background pixels.

Step 5. Apply morphological erosion for *inv_bw(i,j)* with quite big radius of morphological element and write the result in *big_erode_bw(i,j)*. And final binary image is calculated as *final_bw(i,j)=inv_bw(i,j) XOR big_erode_bw(i,j)*.

Step 6. Form final image for segmentation. We create new image: for white pixels in *final_bw(i,j)* we write source color; for other pixels we set up black color.

Algorithm 1. Preprocessing

The result of preprocessing step you can see in Fig.7

Astrocytes detection

Firstly, we apply binarization using simple thresholding. The value of threshold is $(200_r, 80_g, 200_b)$. After that we find all circle contours

on binary images and calculate mass centers for every of them. This mass centers are locations of astrocytes.

First classification of neurons

Step 1. Apply morphological erosion. The size of morphological element is *r*. The shape of structural element is circle. We get *bw_for_neurons_seg(i,j)* image.

Step 2. Find contour on *bw_for_neurons_seg(i,j)*, calculate mass centers for every contours *(xc,yc)*. This is cell centers. Do following for every center:

    Step 2.1. Calculate average intensity (*av_value*) for *(xc,yc)* in *r* radius.

    Step 2.2. Classify center as pyramidal neuron center if *abs(av_value – pn_average)<abs(av_value – sn_average),* where *pn_average, sn_average –* mean intensity value for pyramidal and stellate neurons. Otherwise, classify it as stellate neuron.

    Step 2.3. Draw circle with center in *(xc,yc)* and radius *r* on *first_seg* image. Use green color for pyramidal neurons and red color for stellate neurons.

Algorithm 2. Main part for neurons start classification

On this stage we find cells centers firstly. We also get estimation of the radius of a cell. Secondly, we classify found objects on two types: pyramidal and stellate neurons. It's preclassification for layer detection only. We do steps from Alg.2 for all *r* from *max_neuron_radius* to *min_neuron_radius* with *step_for_neurons* downstep.
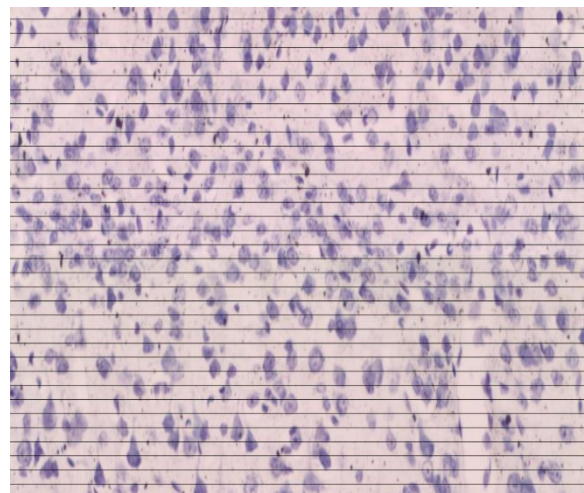

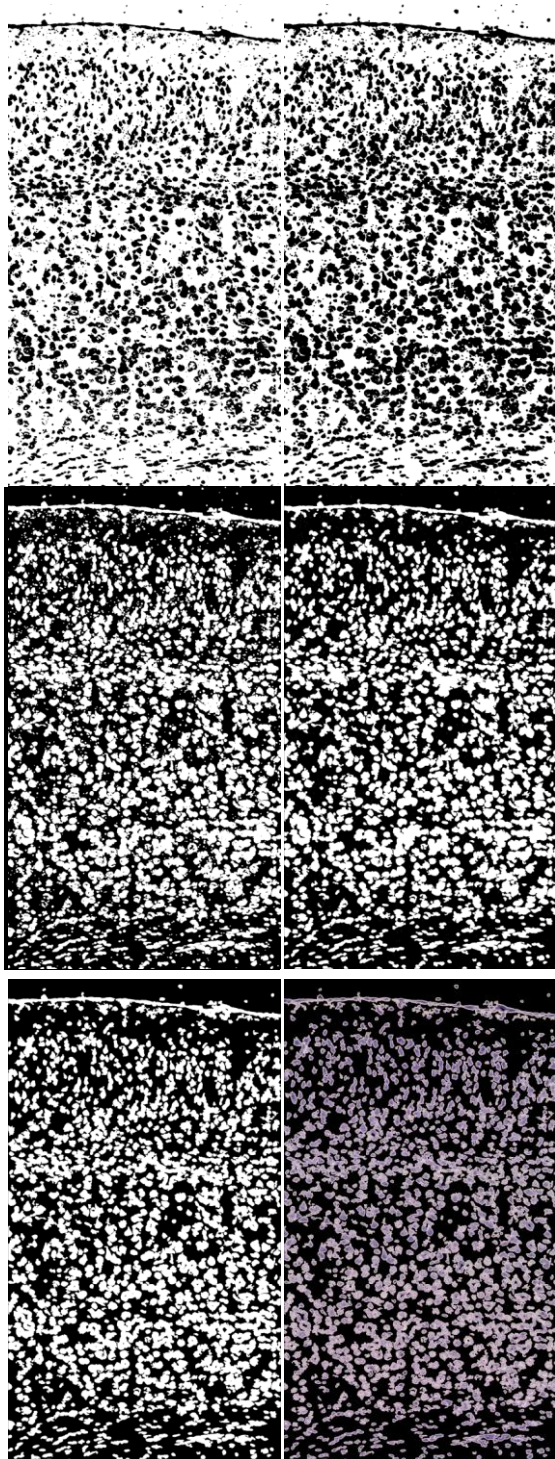Figure 6: Bin separators (1 bin = 25 source lines) for layer detection

Figure 7: Steps 2-7 of preprocessing algorithm.

The result of preclassification algorithm you can on the left image of the Fig.8. In the Fig. 9 you can see results for cells detection and neurons preclassification for different data slices.
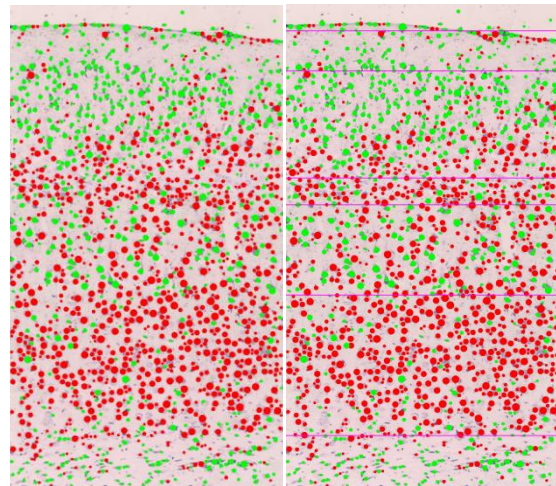
Figure 8: First neuron classification and layer detection based on it.
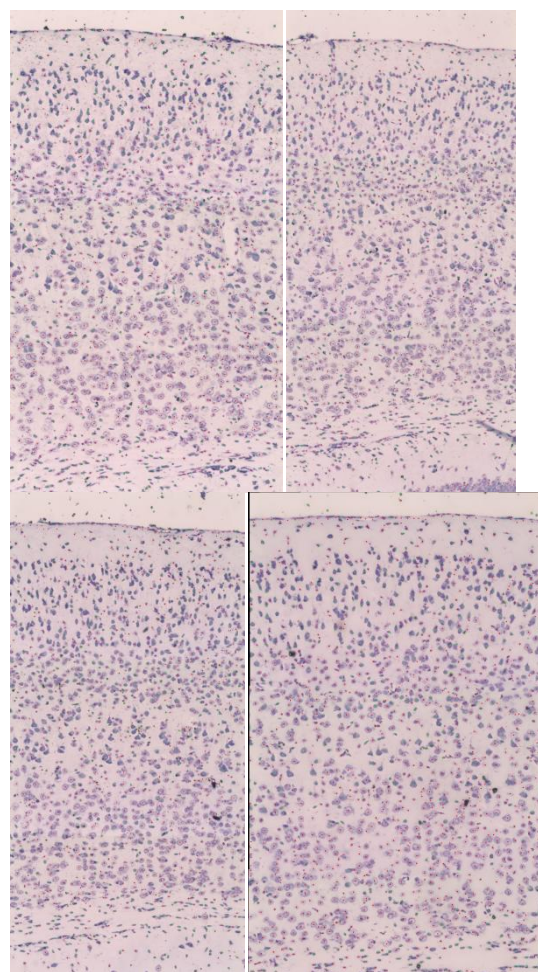

Figure 9. Cells detection and preclassification results for different data slices

Layer Detection

This stage include following two steps. The first is a calculation of cells density for quantized image by lines (Fig. 6). The algorithm for this step you can see in Alg.3. You can see density results on the Fig.9. The second is layer decomposition based on cells

density distribution. To cortex segmentation by layers we need value for six separators: *l0,l1,l2,l3,l4,l5*. We use follow rules for separators settings which were described in Alg.4. You can find cortex layer decomposition on the right image of the Fig.8.

Step 1. Do quantization by source image rows. In every bin we have 25 lines, there are 145 bins summary.

Step 2. For every bin:

Step 2.1 calculate density of pyramidal neurons as:

$$PND = \frac{pixel\_PND\_count}{pixel\_count}$$

where *PND* is a pyramidal neurons density; *pixel_PND_count* is a number of green pixels in a bin of *firt_seg_image*; *pixel_count* is a number of pixels in a bin.

Step 2.2. Similarly calculate density for stellate neurons.

Step 2.3. Calculate summary cells density in a bin.

Step 2.4. Average density data using average box filter (with radius 5)

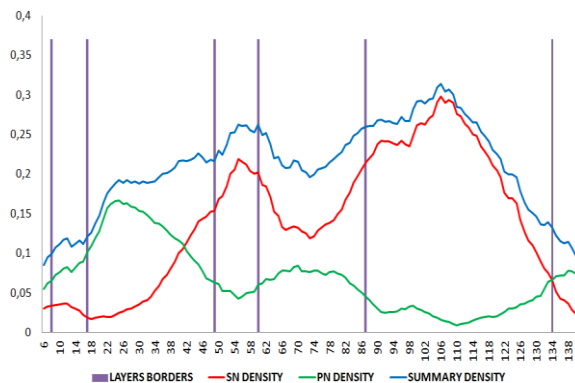Algorithm 3. Algorithm for cells density calculation

Figure 10: Density of pyramidal, stellate neurons and summary density in bins(1 bin = 25 source lines).
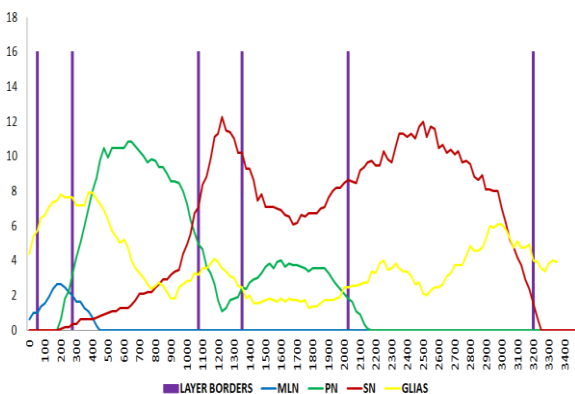

Figure 11: Average cells count along source lines.

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l0 is the start of the molecular layer (layer I). *l0* has value as the first *i* in which *av_summary(i) >= level1*. *level1* is a changeable parameter.

l1 is end of layer I and start for layer II-III. *l1* has value as the first *i* in which *av_summary(i) >= level2 && i>l0*. *level2* is a changeable parameter.

l2 is end of layer II-III and start for layer IV. *l2* has value as the first *i* in which *av_sn(i) >= level3 && i>l1*. *Level3* is a changeable parameter.

l3 is end of layer IV and start for layer V. *l3* has value as the first *i* in which *av_sn(i) < level4 && i>l2*. *level4* is a changeable parameter.

l4 is end of layer V and start for layer VI. *l4* has value as the first *i* in which *av_summary(i) >= level5 && i>l3*. *level5* is a changeable parameter.

l5 is end of layer VI. *l5* has value as the first *i* in which *av_sn(i) < level6 && i>l4*. *level6* is a changeable parameter.

Algorithm 4.Rules for layers separation

Reclassification of neurons and astrocytes

Cells reclassification depends from layer number and was described in Alg.5.

LAYER I : If intensity of the cell center pixel is in the range *[min_glia_val, max_glia_val]* the cell is astrocyte. Otherwise, classify this one as molecular layer neuron.

LAYER II-III: If intensity of a cell center pixel is in the range *[min_glia_val, max_glia_val]*, than the cell is an astrocyte. Otherwise, check if the center was classified as stellate neurons:

if *abs((gray(x,y) − pn_average − 20) <= 15)*,

than reclassify this center as a pyramidal neuron.

LAYER IV: If intensity of the cell center pixel is in the range *[min_glia_val, max_glia_val]* the cell is astrocyte. Otherwise check if center was classified as pyramidal neuron. If *abs(gray(x,y) − sn_average + 20) <= 15)*, reclassify this center a s stellate neuron and as astrocyte otherwise.

LAYER V: Relassification is not carried out.

LAYER VI: If intensity of the cell center pixel is in the range *[min_glia_val, max_glia_val]* the cell is astrocyte. Otherwise reclassify cell as astrocyte if it had pyramidal type.

Algorithm 5. Rules for cells reclassification in different cortex layers

Final result of layer decomposition and cells classification you can see at Fig. 12. Summary count statistics you can see in the Tab 4. and in the Tab. 5. Distribution cells along image rows you can see at the Fig.11.As you see, isolate stellate neurons has good quality of detection. In touched stellate neurons

case algorithm detects more center point than there are actually.

4. EXPERIMENTAL RESULTS

	Layer number	I	II-III	IV	V	VI	Sum
MLN	Human	2	0	0	0	0	2
	Auto	3	0	0	0	0	3
PN	Human	0	27	0	10	0	37
	Auto	0	28	0	12	0	40
SN	Human	0	3	12	11	43	69
	Auto	0	12	11	38	34	95
Astr	Human	2	18	6	16	24	66
	Auto	2	12	3	5	14	36

Table 2. Comparison of the auto-segmentation and human-segmentation cells detection results.

	Auto	**Human**	**From article**
Neurons count	67	60	54
Glias count	25	44	33
Summary	92	104	87

Table 3. Comparison results for one cortex column(part of cortex) in our data (auto- and human-detection) with results from [Dav04] (not the same data)

As a result, in layer V we have 3 times more stellate neurons, and in layer VI we have about 20% fewer stellate neurons than actually.

Auto-detected astrocytes are twice smaller than there are actually. The reason is an inadequate contrast of some astrocytes. It's needed to do an additional preprocessing for better results.

The profile of density and neurons count (from the Fig. 10 and Fig. 11) looks similar with profile from [Mey10].

We also compare our results (in absolute values for one cortex column) with results from [Dav04]. In [Dav04] one can find estimation of neuron count. In our work we detect cells and estimate cell type. The result of comparison one can find in the Tab. 3.
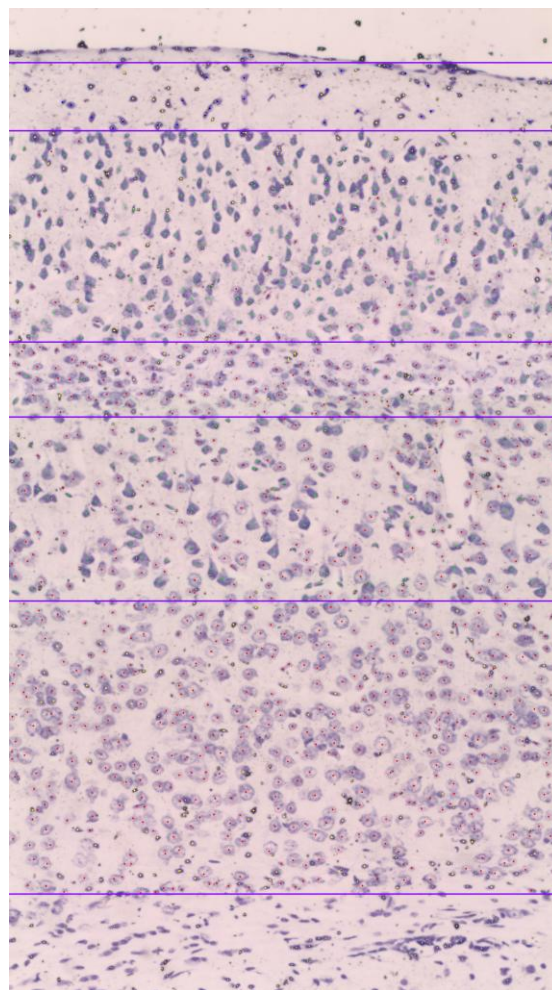

Figure 12. Final neuron and astrocytes centers, and final layer separation.

	Astrocytes	**MLN**	**PN**	**SN**
Layer I	63	49	0	0
Layer II-III	102	0	310	77
Layer IV	39	0	13	123
Layer V	58	0	113	344
Layer VI	113	0	0	358

Table 4. Cells count in one slice.

	Astrocytes	**MLN**	**PN**	**SN**
Layer I	2	1	0	1
Layer II-III	3	0	11	6
Layer IV	2	0	0	7
Layer V	4	0	6	20
Layer VI	14	0	0	15

Table 5. Cells count in one column.

	Detection			
	True positive		**False positive**	**False negative**
	Classification			
	Correct	**Incorrect**		
Summary	731	102	9	52
Astr.	178			
MLN	10		-	
PN	160			
SN	383			

Table 6. Counts of correct (incorrect) cells classification and different types of neuron detection errors

5. CONSLUSION AND FUTURE WORK

Firstly, we propose method for different type cell detection based on morphological operations and different statistics about neuron and astrocytes for all source images (without differences analysis for every cortex layer). Like in [Hey15] and [Kol14] it also depends from the start parameters. Our method allows to get cells localization and estimate size of this cell. The method from [Kol14] allows to get correct edges for every cell. But in [Kol14] there are no classification by neurons type and layers detection. Our method is quite accurate for molecular layer neurons, pyramidal neurons, isolated stellate neurons and astrocytes placed over neurons. Molecular layer neurons and pyramidal neurons have a good quality in the detection and classification. The quality of pyramidal neurons detection is enough for statistics collection, but the quality of stellate neurons detection is not enough. We have good localization results in those layers where there is only one neuron type. But we have inaccurate results in classification of found objects. As future work, we need collect and divide a statistic for every type of neurons by every layer (about color distribution, texture and shape features). It's necessary to analyze a dependence between segmentation quality and noise deleting techniques. Also we need more information about false-positive and false – negative results for neuron and astrocyte segmentation. For correction of localization and segmentation of such results we need to use the same information from nearest slices (source images).

Secondly, we proposed algorithm for cortex layer detection based on detection statistics. All results placed in the article. The proposed algorithm has as good results in layer decomposition as atlas-based algorithms ([All04], [Sen11]).

Thirdly, we got statistics about neuron localization in every layer. For getting statistics and cells features a stereological study is very popular ([Gar12], [Gia12]). In stereological study one has two main steps: get object boundary and get estimation of volume and shape of the object. \Finding boundaries using automatic algorithms (f.e. Canny) may be useless. For our data, Canny algorithm gives inaccurate result for stellate neurons (for both isolated and attached ones). Our algorithm can estimate the location and size of every cell fully automatically.

As the next step we need to add a machine learning algorithm for cell classification. Also it is interesting to check a layer decomposition with initial layer segmentation based on atlas and ray methods. Finally, we should get comparative results with [All04] and [Bra05].

6. ACKNOWLEDGMENTS

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