Computational counting for a quantitative analysis of cells in histologically prepared brain sections

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Abstract
We propose a reliable method for automatic counting of cells in brain sections for different antibodies (NeuN, Parvalbumin, GABA and c-Fos) and Nissl-staining. The images of stained sections are binarized by thresholding. Then regions are clustered using a common clustering algorithm. After choosing only proper sized clusters the detected cell-bodies are counted. The parameters of the algorithm are adjusted manually and remain constant for different probes. The computational cell counting method provides correct counting results, shown by a comparison of computational results and counts gained by human experimenters.

Keywords
cell counting, clustering, neuronal tissue, quantitative analysis, threshold images

1 Introduction
The central nervous system of animals and humans consists of billions of glial and nerve cells. Depending on their developmental and physiological stage, they express different proteins and transmitters, which can be detected by antibodies. A change in the number of labelled cells corresponds to a change in the expression of proteins and transmitters and therefore a change in the function of the neuronal network. To characterize the changes it is essential to know the number of considered cells in each experiment.
The counting of stained cells can be performed directly at the microscope or based on images taken by a camera on the microscope. To gain reliable counting results it is necessary to take many images from areas of several sections for counting. In any case, the experimenter counting the cells has to evaluate noisy data. This noise is induced by staining or by the image

acquisition process. Additional errors occur with each count performed by humans. The manual cell counts include high variances due to different levels of experience in counting, due to variable staining intensity, and due to individual perception. To avoid these problems we applied a straight forward computational method for cell counting, which is calibrated manually including expert knowledge and proven to be reliable for different staining methods.

2 Materials and Methods

2.1 Preparation of Tissue

Sprague Dawley rats (N=24) were sacrificed with an overdose of urethane and their brains were fixed by intracardial perfusion with normal saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PBS, pH 7.4). After a 4-h post-fixation at 4^{o}C in the same fixative the brains were embedded in paraffin. Coronal sections of 5µm were cut serially and every tenth section was placed on gelatine-coated slides, dewaxed in xylene and transferred through a descending ethanol series into PBS and stained with cresyl violet (a Nissl stain). The adjacent sections were treated immunohistochemically with antibodies against NeuN (neuronal nuclear antigen; 1:1000, Chemicon), GABA (1:4000, Sigma), Parvalbumin (1:1000, clone, PA-235, Swant) and c-Fos (1:3000, Dianova). For details see [1].

2.2 Image Acquisition

Images of stained sections were acquired using a colour video camera (CCD KH 604, Heimann; Metamorph, Universal Imaging) on an Axioskop (Zeiss, Germany) at magnifications of 50-200x.

2.3 Counting Method

The counting was performed on grey value images (value range = [0,255]). The process of counting is separated into two modes: calibration and counting. In the calibration mode the parameter of the algorithm can be adapted according to the staining method and cell size. In the counting mode the probe is then analysed using the calibration settings and the detected cells are automatically counted.

2.3.1 Calibration

In the calibration mode thresholds for the considered grey values in the images and for a proper size for acception of clusters are adapted. The calibration is achieved by manually counting three images of each animal and marker. With the counted areas the thresholds of the program are set. Using this method expert knowledge can be directly induced into the evaluation. In the calibration mode four parameters of the algorithm are set.

- setting thresholds for binarization (T_{min}, T_{max})
- setting cluster size (s_{min}, s_{max})

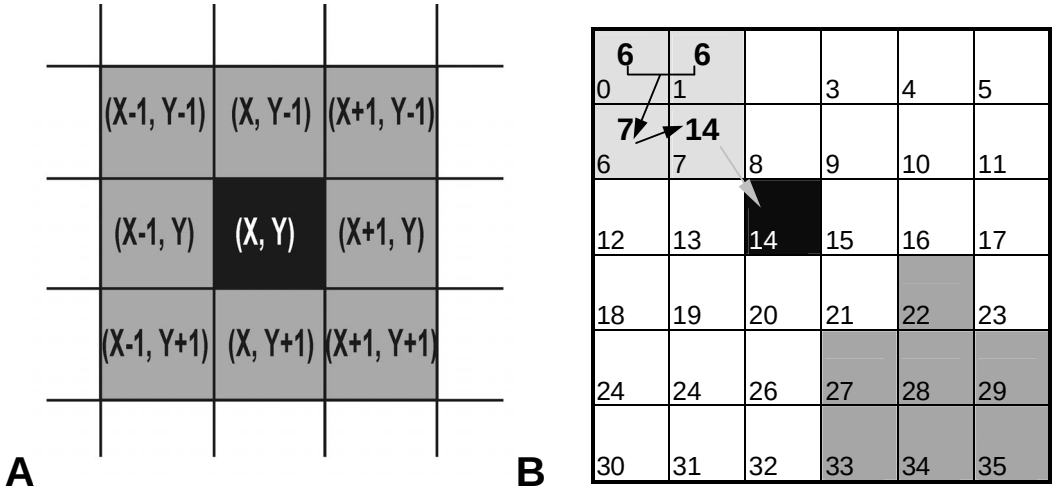

Figure 1. Considered adjacent pixels in the applied clustering algorithm. A: The central pixel at position (x,y)(black) is compared with its eight neighbouring pixels. B: If adjacent pixels are set in the neighbourhood (here shown in bright grey for the analysed cluster), the current pixel and neighbouring pixels get organized into a tree with the index of its root serving as an identification during the first pass. This is done by letting each set pixel point to its parent in the tree. The small numbers show the pixel number. The dark grey region characterizes a not yet labelled, disconnected cluster.

Binarization In order to detect the relevant cell bodies the grey value image g(x,y) (x is the image row and y the image column) is transformed into a binary image b(x,y) by thresholding.

$$b(x,y) = \begin{cases} 1 : T_{min} \le g(x,y) \le T_{max} \\ 0 : \text{otherwise} \end{cases}$$

By applying a lower threshold T_{min} very dark regions are eliminated (low grey values), like borders of the tissue. An upper threshold T_{max} serves for elimination of brighter background. The binarization is applied to separate stained regions (b(x,y) = 1, cells) from unstained regions (b(x,y) = 0) in the image.

Clustering After binarization, the regions with a value of 1 in the binary image (b(x,y)) are clustered by labelling connected regions [4], resulting in a cluster image c(x,y). In this applied clustering method, each set pixel in the image is joined into one region with all adjacent set pixels in the eight neighbouring pixel positions (Fig. 1). Two source image pixels $b(x_1,y_1)$ and $b(x_2,y_2)$ are considered adjacent if

$$|x_1 - x_2| \le 1 \wedge |y_1 - y_2| \le 1$$

If two source image pixels are set and adjacent, the corresponding cluster image pixels will get assigned the same non-zero region label. The clustering algorithm

uses two passes on the binary image: in the first from left to right, forward pass, the connectivity of the regions is established by organizing all set pixels in a connected region into a tree where the index of the root is identifying the region. The second, backward pass then assigns consecutive region numbers to elements of separate clusters.

2.3.2 Counting

To detect only clusters of proper size, the histogram h(c(x,y)) of the cluster image is determined. In the histogram the number of pixels of each cluster index is stored. This number represents the size of a cluster. The more pixel of one value exist the greater is the according cluster. Two parameters determine the minimal (s_{min}) and maximal (s_{max}) cluster size for acception. Those size considerations are necessary due to small disturbances or effects of tissue-borders. Improper clusters are ignored and the remaining clusters are counted.

3 Results

The reliability of the algorithm is proven by a comparison of computational results and counts gained by 18 humans observers. For this comparison 15 images from different probes and different magnifications were counted automatically and by histologists.

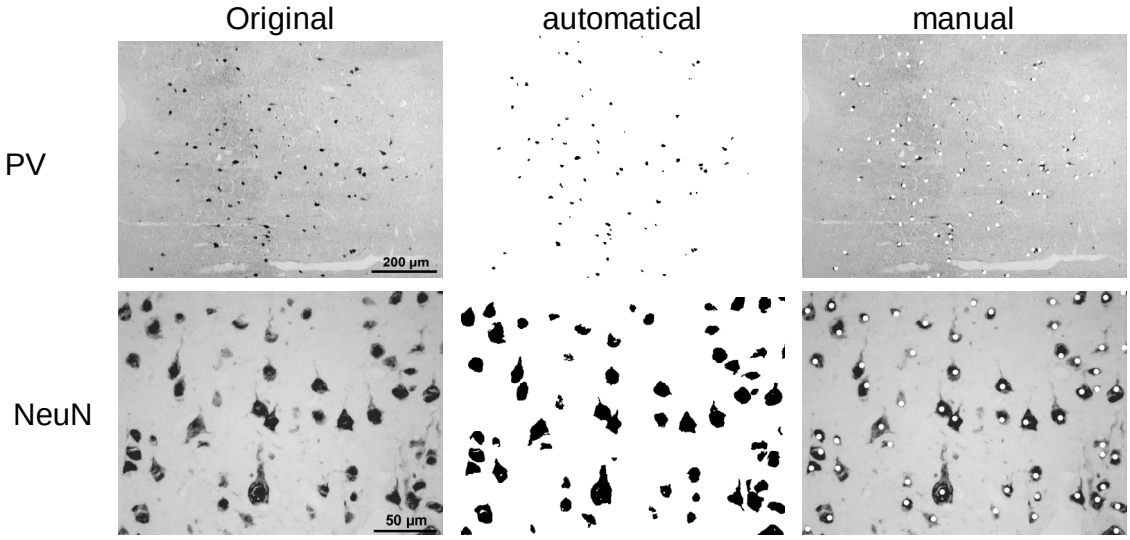

Figure 2. Images of sections (first column) stained with Parvalbumin (PV) and NeuN. In the second column are the binarized images with the accepted and automatically counted cells presented. The third column shows the manual counted cells marked by dots. Magnification see scale bars in original images.

The examples in Figure 2 show that the program counts for different markers just as good as the histologists.

The results of the comparison for different magnifications are shown in Figure 3. A linear relationship between manual and automatic counts exists with a correlation coefficient of 0.93 (Fig. 3). The results of the automatic counting fall within the standard deviation of the manual counting (Fig. 3). This holds for low and high densities of cells in the images, independently of the used marker and magnification. The analysis points out that the results of the automatic counting are close to the mean value of the manual counts for each image. A further advantage of the automatic counting is the fact that in the cell counting process the cell size is simultaneously determined by the histogram. By manual counting the cell size would have to be measured for each cell separately.

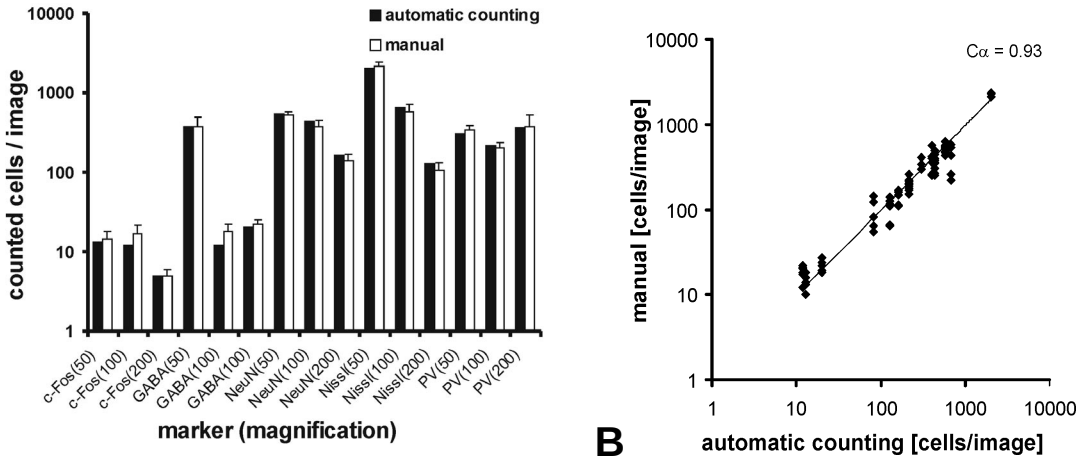

Figure 3. Comparison of counting results of automatic vs manual. A: summary of counting results for all 15 evaluated images of automatic counting and mean values and standard deviations of manual counts. B: linear correlation between automatic and manual counts. Each symbol represents the counting result of an image. A value on the x-axis corresponds to several values on the Y-axis due to the counting of the same image by several histologists.

4 Conclusions

Counting cells in histologically stained sections is time-consuming and manual counting is often a tedious work with highly error-prone results. By the proposed computational algorithm the counting method produces reliable counting results, which show a high consistency, invariably the same counts are obtained for a given image, which is not the case if counts are performed by humans. Furthermore the algorithm can be used for different markers and different magnifications. Besides this program there are many other programs for analysis of biological data developed by researchers [2, 3, 5, 6, 7] or companies (products like MetaMorph, KS 300 or ANALY*SIS*). Most of those programs are rather specific for one application and require a good insight to adjust them for other purposes. Commercial programs normally have a wider range of applications, but most of them are very expensive and need a long training period. Additionally the user is not informed about details of the algorithms which are

used for analysis. In contrast to these the used algorithm is easy to understand and can be easily adjusted to further purposes.

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