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

A. Benali[*+], *I. Leefken*[+], *E. Weiler*[*]

Ruhr-Universität Bochum
[+] Institut für Neuroinformatik, Lehrstuhl für theoretische Biologie
[*] Institut für Physiologie, Abteilung für Neurophysiologie
Bochum, Germany

Abstract (100 words)

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 thin 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 remain constant for different probes. The computational cell counting method provides correct counting results which is shown by a comparison of computational results and counts gained by human experimenters.

Summary (1000 words)

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, usually detected (in immunohistochemical techniques) by antibodies. A change in the number of these stained cells corresponds to a change of expression of proteins or transmitters and therefore a change in the function of the neural network. To characterize these changes it is essential to know the number of considered cells under different physiological and experimental conditions. To gain reproducible counting results, images are taken from areas of several coronal sections. The images are noisy. This noise is caused by the staining process, by individual differences of the animals, by anatomical differences of the cortices (several cortical areas respectively the layers contain different densities of cells), and by the image

acquisition process. Additional errors occur with each count performed by humans. The manual cell count includes 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 can be adapted manually and which proved to be reliable for different antibodies and nissl-staining.

Preparation of tissue

30 Sprague Dawely rats 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 (pH 7.4). After a 4-h post-fixation at 4°C in the same fixative the brains were embedded in paraffin. Coronal sections of 5μm were cut serially and each section was collected. Every tenth section was placed on gelatine-coated slides, dewaxed in xylene and transferred through a descending ethanol series into PBS (phosphate buffer solution). For cytoarchitectural control alternate sections were stained with cresyl violet (a nissl stain). The adjacent sections were treated immunohistochemically with antibodies against NeuN (neuronal nuclear antigen), GABA (gamma-aminobutyric acid), Parvalbumin and c-Fos and visualized by DAB. All experiments conformed to NIH guidelines and were approved by the German Animal Care and Use Committee.

Image Acquisition

Results of the nissl-stained sections and immunohistochemical experiments were obtained using an Axioskop (Zeiss, Germany) and a Leitz Wetzlar Dialux 20 microscope (Leica, Germany) equipped with a color video camera (CCD KH 604, Heimann; Metamorph, Universal Imaging). Cell counts were made at a final magnification from 50x-200x. For the investigation of the marked cells at least 9 coronal-sections with a rostro-caudal distance of 100-250μm were evaluated for each marker. In all coronal sections each hemisphere was divided into six areas with an extension (medial-lateral) in layer 4 of 500μm.

Counting Method

The counting was performed on grey value images (value range = [0,255]). The process of counting is separated into two modes: counting and calibra-

tion. In the counting mode the probe is analyzed and the detected cells are automatically counted. In order to count the number of relevant cell bodies the grey value image is transformed into a binary image by thresholding. Each pixel with a grey value that falls into a range of grey values (determined by manual adaption of a lower and a higher threshold) is given a value of 1, otherwise a value of 0 ($\rightarrow$ binary image). The comparison with a lower threshold results in an elimination of low grey values, like borders of the tissue. An upper threshold serves for elimination of brighter parts of the image.
Next, the regions with a value of 1 in the binary image are clustered by a common clustering algorithm. In this applied clustering method each pixel-value in the image is compared to all of its adjacent pixels. If adjacent pixels are set this cluster of pixels is associated with an index characterizing the cluster. The cluster image is determined by performing the algorithm twice on the binary image. First in forward direction and then vice versa to cope with adjacent clusters.
Then the histogram of the cluster image is evaluated to accept clusters fulfilling the condition that the region is neither too big nor too small. Two parameters determine the minimal and maximal cluster size to be accepted to avoid either small disturbances or effects of tissue-borders. Having performed those operations the accepted clusters are counted.
In the calibration mode the thresholds for the grey value image and for the cluster size are adapted manually. This calibration is achieved by manually counting three cortical areas from each animal and marker. With the counted areas the thresholds of the program were set. Using this method expert knowledge can be directly induced into the evaluation.

Results

The reliability of the algorithm is proven by a comparison of computational results and counts gained by human observers. For a comparison of the algorithm and human counts 10 images from different probes were counted by both. For all samples the computational results fell within the standard deviation of the human experimenters and were close to the medium over all human counts. The human experimenters as well as the computer were faced with different samples. The samples were taken from different animals, from probes prepared with different antibodies (NeuN, Parvalbumin, GABA and c-Fos), nissl-staining, and from different image magnifications (50x - 200x).

Parts of the samples were counted more than once to regard the variance in counting results of single humans.

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. Additionally the time-consumption of the counting is highly reduced.