Cellular and subcellular co-localisations of immunologic expression patterns revised by Boolean feature operators

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Abstract. Modern Confocal Laser Scan Microscopy is a sophisticated technique allowing acquisition of information from different fluorescence markers in the same tissue by separating them into different confocal channels. For an adequate interpretation of these multidimensional images advanced image processing techniques are required. In this study, we introduce an automated image analysis based on Boolean logic working with features instead of single pixels. Feature based image analysis preserves the original morphology of the objects and allows the unlimited identification of co-localisations. We demonstrate the practicability of our feature-based algorithm on triple-immuno fluorescence stained neural cells of the auditory cortex of gerbils.

1 Introduction

Modern Confocal Laser Scan Microscopy (CLSM) provides information from different markers separated into different confocal channels with high spatial resolution. Moreover, due to sophisticated data acquisition schemes (Multiple Image Stack Acquisition, MISA [1]) it is possible to investigate structures with subcellular resolution at a macroscopic level of histological specimens in one dataset resulting in huge multidimensional data spaces. Manual analysis of such datasets is extremely time consuming and therefore impracticable. Consequently, a robust (due to the naturally high noise and variability in biological specimens) and automated (due to the large datasets) image analysis is necessary.One major topic of interest in cell biology today is the co-localisation of different cell properties (different morphology, different protein or gene expression) in one specimen e.g. as a hint for a possible interaction of these labellings. Multichannel scanning provides the ability to record cells with many different specific markers in a given histological section at one time. Conventional co-localisation is performed as an additive mixture of the different colour channels in a given colour model such as the RGB space and therefore limited to the colour space dimensions. Moreover, only overlapping pixels are recognized instead of whole objects. This leads to a loss of the original object geometry. Consequently, measurements beyond a simple quantification of the coloured pixels are impossible.

As a new suggestion this paper examines feature-based image analysis such as Boolean logic operations instead of single pixels in order to overcome the above described problems.

2 Material and Methods

2.1 Histological material

As specimens we worked on immuno stained gerbil brain sections. Focus of interest was the differential expression of proteins (the calcium binding protein parvalbumin (PV) and the immediate early gene product c-Fos) in the auditory cortex after a shuttle box learning paradigm [2]. The production and the staining of the biological sections were made according to standard procedures [2] and will not be described here. In order to get information about the total number of cells the sections were counter-stained with propidium iodide (PI) solution.

2.2 Image acquisition

Areas including the auditory cortex were scanned with a confocal laser scanning microscope (Leica TCS 4D) equipped with a Krypton-Argon-Ion laser (488/568/647 nm). A complete scan of one section – one multi-image-stack - consisted of 7 (x) x 7 (y) x 5 (z) images x 3 channels (FITC – c-Fos, CY5 - PV, propidium iodide), i.e. 734 single images.

2.3 Image processing

For an adequate automatic image processing it is necessary to establish an object model which can be defined by the user. In our case these parameters are a given size and grey value range. Based on such a specific object model the objects of interest can be distinguished from the background.
Image processing algorithms were programmed on a standard PC in IDL (©RSINC) language. The initial image processing consisted of an auto-contrast enhancement algorithm. Subsequently a 3x3 median filter and a 7x7 octagonal grey scale Opening were applied. Background elimination, which is most important for biological specimens, was done by the elimination of the lower grey levels at a certain threshold. For definition of this threshold we developed an automatic procedure based on the histogram type and value distribution. The histograms were found to consist of two types. For histograms with a single sharp peak, the threshold is set in the beginning (i.e. after contrast enhancement) and the value equates the 90% quantile of the histogram. Consequently, for broad histograms the threshold is set at the end of the processing (i.e. after grey scale opening) and the median of the equalized histogram was chosen as value. The pre-processed images were binarized using an auto-threshold procedure [4] which performed comparable to Otsu's thresholding [5] but is much faster. Finally, touching objects were separated by watershed segmentation.

2.4 From pixels to features

Features are connected pixels that are treated as a whole. Grouping pixels into features was done on binary images starting with a connected component analysis [6]. Next, the grey values of each feature are obtained from the original grey values image with a

mask operation. Once all features are established in an image, it is easily possible to exclude objects outside the object model. Smaller objects are certainly background particles and larger ones may be not separated cells. Objects which are to dark may be unspecific background staining. The algorithm for Boolean logic with features was programmed in IDL according to Russ [7]. As one example, the principle of a Feature-AND is that an entire feature – group of connected pixels - in one of image is kept if any part of this feature touches a feature in an other image.

3 Results

Immuno-staining of the very same cell with different fluorescence dyes let the cell shapes appear differently (compare Fig. 1), which leads to wrong results when cell types are compared according to their shape. To avoid this, it is necessary to compare the cells relying on one dye, giving the geometry of all cells independent of their functional labelling. In the case of this study this dye is propidium iodide (PI). A PI stained section was related separately to the same section stained with c-Fos and parvalbumin (PV) by the application of Feature-AND. Fig. 1D shows such a combined image.

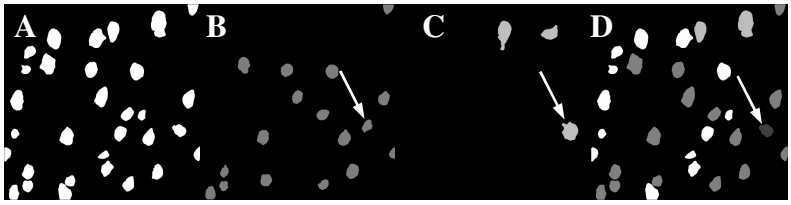

Fig.1 Segmented cortex cells. A: total cells, PI stained; B: c-Fos labelled cells; C: PV stained cells. D: C co-located cells from A, B, and C by feature-AND. The arrow indicates a PV and c-Fos double labelled cell.

The investigation of co-localisation using Feature-AND leads to the evidence for cells that are double-labelled with c-Fos and PV (Fig. 1, Fig. 2). This is a new finding which has been not described so far by manual analysis. Also shown in Fig. 2 is the influence of the order in which the images were analysed by Feature-AND. Fig. 2C and 2D show the same cells, double-labelled with c-Fos and PV, but they look very different. In C a feature-AND was applied in the order c-Fos- AND PV, in D vice versa resulting in different resulting geometries determined by the first operand.

Once the co-localisation is analysed, it is possible to compare co-localised cell to not co-localised cells on the basis of the same staining and therefore on the same geometry. As an example for the procedures described above we analysed cortical cells with in total 16040 c-Fos-stained cells 4070 PV-stained cells. We found, that 1240 of them are co-localised. That means, that 7% of the c-Fos-cells and 30% of the PV-cells are co-localised with each other.

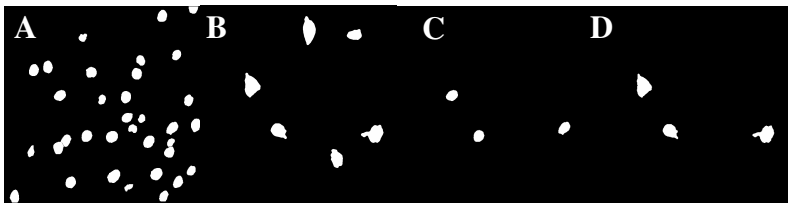

Fig. 2 Feature-And of c-Fos and pv stained cells. A: c-Fos stained cells. B: PV stained cells. C: c-Fos feature-AND PV. D: PV feature-AND c-Fos.

4 Discussion

In this study, we developed an automatic procedure for the segmentation of fluorescence stained cells in CLSM-images tested on neural cells of the auditory cortex of gerbils. Furthermore, we established a feature-based analysis strategy in order to deal with whole objects resp. cells instead of single pixels. The proposed algorithms work fast and robust. The new co-localisation analysis revealed new types of especially very seldom co-localisation which have not been described so far based on manual analysis (c-Fos and PV co-localisation). In summary, the main advantages of these feature-based operations are that (1) the original geometry of each objects is preserved, (2) there is no limit in the number of co-localisation patterns. Further investigation of function, absolute and relative abundance, morphology and distribution of cells and the corresponding co-localisations of immuno labelling patterns within the cortex can be now done automatic, fast, and efficient.

5 References

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