



Automated high through-put colocalization analysis of multichannel confocal images

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Received 27 August 2002; received in revised form 8 April 2003; accepted 20 April 2003

KEYWORDS

High-through-put;
Colocalization;
Analysis;
Confocal microscope;
Matlab

Summary The laser scanning confocal microscope (LSCM) generates images of multiple labelled fluorescent samples. Colocalization of fluorescent labels is frequently examined. Here we present an example where localization of fluorescent analogues of cloned protein were referenced to fluorescent antibodies directed against the proteins of cellular compartments. Colocalization is usually evaluated by visual inspection of signal overlap or by using commercially available software tools, but there are limited possibilities to automate the analysis of large amounts of data. We developed a simple tool using Matlab to automate the colocalization procedure and to exclude the biased estimations resulting from visual inspections of images. The script in Matlab language code automatically imports confocal images and converts them into arrays. The contrast of all images is uniformly set by linearly reassigning the values of pixel intensities to use the full 8-bit range (0–255). Images are binarized on several threshold levels. The area above a certain threshold level is summed for each channel of the image and for colocalized regions. As a result, count of pixels above several threshold levels in any number of images is saved in an ASCII file. In addition Pearson's r correlation coefficient is calculated for fluorescence intensities of both confocal channels. Using this approach quick quantitative analysis of colocalization of hundreds of images is possible. In addition, such automated procedure is not biased by the examiner's subject visualization.

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1. Introduction

The laser scanning confocal microscope (LSCM) is routinely used to acquire images of single-, double- and triple-labelled fluorescent samples. Images of thin optical sections of fluorescent specimen are generated. Each picture element (pixel) represents a small volume in the sample. The colocalization

of the pixels is frequently examined in specimens stained with multiple fluorescent probes [1]. For example, a fluorescent analogue of protein may be referenced to fluorescent probes for cellular compartments or structures. The use of red, green and blue (RGB) colours is most informative for displaying the distribution of up to three fluorescent probes labelling the cell.

Many authors analyze fluorescent signal overlap by visual inspection. In a three-color image, the colocalization appears as a different additive colour [2]. For example colocalization of green and red pixels attains yellow appearance. The visual

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inspection is a quick and intuitive method, but it is not quantitative. A tool for colocalization assessment is built into several acquisition and analysis software packages. A threshold criterion may be selected in a pixel correlation plot or by selecting the threshold levels numerically for each channel. The fraction of colocalized pixels for any threshold level may be calculated from such an analysis. In order to set consistent threshold levels, the image contrast has to be adjusted prior to analysis. Ideally, image intensities should use the full 8-bit range (0–255). To avoid adjusting the contrast in reference to a single pixel with maximum intensity value, the images should be filtered appropriately prior to determination of the minimum and maximum pixel intensity values.

Manipulation of images using commercially available software tools requires extensive user intervention. In biological systems there is often some degree of variability between the images from the same or different preparations, which sometimes requires large numbers of images to quantitate and compare different sets of images. Here we have developed the computer routine to automate the evaluation of colocalization of fluorescent probes from confocal images.

2. Materials and methods

2.1. Cell cultures isolation, immunocytochemistry, introduction of plasmid DNA into cells and confocal microscopy

Anterior pituitary cells were obtained from male Wistar rats by enzymatic dispersion; this was enriched for somatotrophs as described previously [3] and seeded onto coverslips.

The plasmid DNA encoding fusion proteins between the marker proteins and the green fluorescent protein (GFP) was introduced into 24 h old cells by cationic lipids (Lipofectamine Plus™ reagent, Life technologies), according to the manufacturer's instructions. After 24 h the cells were prepared for immunocytochemistry.

Somatotrophs were washed in PBS, and then fixed for 15 min in 4% paraformaldehyde in PBS. For the following 10 min cells were kept in fixative containing 0.1% Triton X-100 and then four times washed with PBS. Non-specific staining was reduced by incubation of cells in 3% bovine serum albumin (BSA) and 10% normal goat serum in PBS. Cells were then incubated with primary antibodies for 2 h at 37 °C, diluted 1:100 in PBS containing

3% BSA. Cells were then washed and incubated in PBS containing Alexa 546-labeled *anti*-mouse secondary antibodies (Molecular Probes, Netherlands) and 3% BSA for 45 min, washed and mounted in Light Antifade Kit (Molecular Probes, Netherlands).

Cell images were acquired on a Zeiss LSM 510 confocal microscope with an x40 oil Plan-Neofluar objective (NA 1.3; Carl Zeiss). Fluorescent dyes (GFP and Alexa 546) were imaged sequentially in a frame-interlace mode to eliminate cross talk between the channels. GFP was excited with the 488 nm line of an argon laser. Emission was monitored using a 505–530 nm bandpass filter. The Alexa 546 was excited with the 543 nm line of He/Ne laser. The emission signal was filtered by a 560 nm long-pass filter.

3. Results

To automate the determination of colocalization of fluorescent labels in optical slice we developed the software tool using Matlab (Mathworks, Natick, MA). Matlab is a high-level language for technical computing, where an array is a basic data element and is therefore especially well suited for basic image analysis. It may solve image-computing problems quicker than it would have taken to write a program in a scalar language such as C or Fortran. The image processing was performed on a PC computer with Microsoft Windows 98 operating system.

We first exported confocal images as 8-bit tag image file format (TIFF) files. Images were cropped before the analysis when the signal was also outside the area of interest. The script in Matlab language code automatically imports the images and converts them into arrays (two dimensional image with three colour channels as the third dimension).

The pixel intensities in acquired confocal images often do not cover the available brightness range completely. This is to avoid the pixel values at the dark and/or light ends of the range to exceed the digitalization and storage capacities and hence to prevent the clipping of pixel values, which would result in the loss of information [4].

To compare images with different acquisition settings, the program stretches the contrast of each channel in order to use the full range of pixel intensities (0–255; Fig. 1B, Fig. 2B). The values of pixels are reassigned in this procedure. The mapping is linear. Before determining the darkest and the lightest pixels the original image is filtered. Each pixel intensity is averaged with pixel intensities of all eight neighboring pixels. The darkest pixels in the original image are assigned to zero,

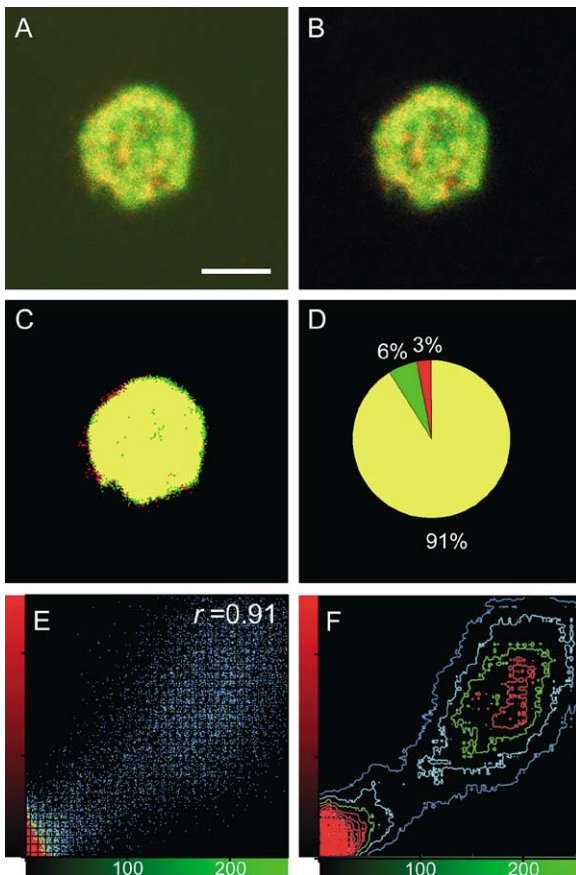


Fig. 1 The software output in the case of complete colocalization of fluorescent markers. During the procedure of automated colocalization the software is displaying the input image (A), the contrast-adjusted image (B), the mask of the pixels above the threshold in one or both (colocalized) channels (C), the pie chart of corresponding level of colocalization (D), the pixel correlation plot (E), and the contour plot (F). The threshold level for this display (A–D) was set to value 51, which corresponds to 20% of the maximal brightness level. The fluorescence intensities of the pixels in correlation and contour plots are colour-coded in blue–cyan–green–yellow–red for increasing intensities. r represents Pearson's correlation coefficient calculated for both confocal channels. Horizontal and vertical scales (in E and F) represent fluorescence intensity value of green and red confocal channel, respectively. The cells were labelled with the fluorescent fusion protein between the mitochondrial marker cytochrome *c* and green fluorescent protein (Cyt*c*-GFP, green, [5]) and the red-labelled antibody, which recognises the cytochrome *c*. Scale bar=5 μ m.

the lightest pixels are assigned to 255 and the intermediate values in the original image are given new linearly interpolated values.

The next step in automated procedure is binarization of each channel of the original image based on several threshold levels, in which the 256 grey levels in the contrast-adjusted image are mapped

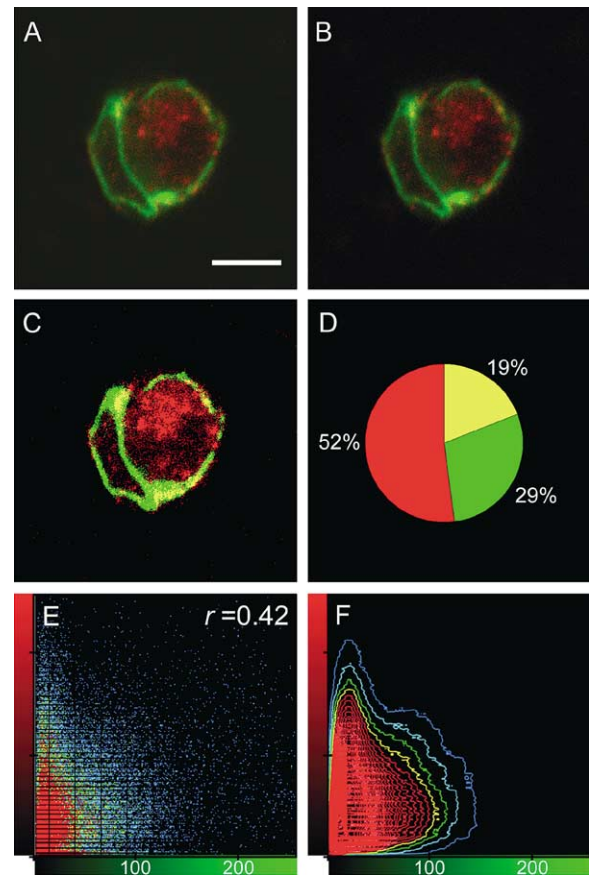


Fig. 2 The software output in the case of non-colocalized fluorescent markers. The layout of the images displayed is described in Fig. 1. The threshold level was set to the value 51, which corresponds to 20% of the maximal brightness level. The cells were labelled with the fluorescent fusion protein between the endoplasmic reticulum marker Sec61 and green fluorescent protein (Sec61-GFP, green) and the red-labelled antibody, which recognises cytochrome *c*. Scale bar=5 μ m.

into a two-level scale, with 0 (below threshold) and 1 (equal or above threshold). Software sums up all pixels above certain threshold levels for each channel and those pixels that are above thresholds in all channels.

During the calculation procedure, the current input image, the contrast-adjusted image, the mask of pixels above threshold in one or both (colocalized) channels, and a pie chart of corresponding quantity of colocalization are displayed (Figs. 1 and 2). The fractions of colocalized pixels relative to the first, the second or both confocal channels are calculated from every image and saved into an ASCII file.

In images, where fluorescent signal is not distributed in all-or-none manner, analysis of correlation between channels may be more appropriate to determine colocalization. In addition to the

method based on thresholding, the software is also calculating Pearson's r correlation coefficient for fluorescence intensities of both confocal channels, and P -values for testing the hypothesis of no correlation.

Here we demonstrate that the presented automated high through-put colocalization analysis may be used to objectively analyze many confocal images at certain threshold value, which is determined arbitrarily based on positive and negative control experiments. As an example we show here the analyses of the two sets of data. In a biological experiment these would represent the two control cases, i.e. of the complete colocalization, where we have used two different fluorescent markers for the same cellular target (Fig. 1) and when the two fluorescent markers are not colocalized (Fig. 2). Such controls are necessary for determining the uniform threshold level, which is then used for comparing the levels of colocalization of unknown markers throughout the experiment. In the case presented below, we determined the optimal threshold value to be 51, which corresponds to 20% of the maximal brightness level. We compared the distribution of fluorescent antibody labelled cytochrome c (red) with the fusion protein between cytochrome c and green fluorescent protein (Fig. 1). In this example we found 91% of colocalized pixels (Fig. 1D, range from 65 to 92%). The correlation coefficient r for fluorescence intensities of both confocal channels (Fig. 1E and F) was 0.91, ranged from 0.68 to 0.94 ($N=13$). In the second case (Fig. 2) we compared the distribution of the two unrelated markers by fluorescent antibody labelled cytochrome c (red) and the fusion protein between the endoplasmatic reticulum marker Sec61 and green fluorescent protein. In this example we found 19% of colocalized pixels (Fig. 2D, range from 17 to 60%), and the correlation coefficient 0.42 (Fig. 2E and F, range from 0.26 to 0.77, $N=10$). The pixel correlation plots (fluorograms) and contour plots provide a representation of the degree of correlation between the channels for each image, whereas cumulative pixel correlation plots and cumulative contour plots represent the correlation for the series of images (not shown). The cumulative pixel correlation plots may be used as an aid for choosing adequate threshold value, which would separate background fluorescence from the signal.

To summarize, all images from the source directory are automatically imported into the program and are sequentially analyzed. The resulting counts of pixels above several threshold levels in any number of images are saved into an ASCII file. The analysis of one image of 512×512 pixels takes 4 s of

computing time on a standard PC Pentium III computer. If the thresholding was scalar-based instead of array-based, it would take 155 s per image.

4. Discussion

The colocalization of different fluorescent probes is frequently examined in the biological samples. The analysis is often performed by visual inspection of the fluorescent signal overlap. Colocalization is observed as a different additive colour, e.g. an overlay of green and red colour results in yellow [2]. Determining the degree of colocalization by visual inspection only is difficult in biological samples, because of variations, which can occur even within the sample (e.g. when the cells do not take up the equal amount of labels throughout the coverslip). Variations in label intensities are often observed between the experiments although the same reagents are being used. Therefore, it is difficult to objectively perform analyses based on visual inspection or manual adjustments of parameters.

Apart from sample variability, there are other factors, which may contribute to the appearance of co-localization. Confocal microscopy is effective at removing a substantial amount of signal generated by labelled structures located above and below the focal plane of interest; nevertheless the top and the bottom surfaces of the optical section are not absolute. Fluorescently labelled structures above and below the focal plane will contribute some out of focus scatter to any given optical section due to the point spread function [6]. This may lead to false positive colocalization as described by Silver and Stryker [7]. These authors developed an object-based method for measuring the amount of colocalization of immunofluorescently labelled antigens with a population of axons that were labelled with an anatomical tracer. The object-based analysis was well suited for applications in which the antigen of interest labelled punctuate structures. The method required manual selection of boundaries. When labelled structures are not punctuate, pixel based analysis is well suited.

Commercially available software tools also evaluate pixel-based colocalization in various image types, but they require extensive user interaction. The analysis of large collections of images with multiple threshold levels is therefore time consuming and may also lead to biased results.

To quantitatively analyze large collection of images we developed a simple tool using Matlab (Mathworks, Natick, MA). The automated analysis enables uniform, therefore objective analysis of all images, regardless of their appearance. This results in the

numeric output of the number of co-localized pixels and of non co-localized pixels from each fluorescent dye. This not only enables the statistical analysis of the degree of co-localization between the two markers, but also enables comparing the colocalization levels between different markers.

The computer routine presented in this paper automates and quantitates colocalization analysis, with no need of user interaction. The script in Matlab language code automatically imports images and converts them into arrays. The contrast of each channel is automatically stretched in order to use the full range of pixel intensities. The next step in automated procedure is binarization of each channel of the image based on several threshold levels. As a result, counts of pixels above threshold levels in any number of images are saved in an ASCII file. The uniform threshold value is determined arbitrarily based on positive and negative control experiments, i.e. the complete colocalization and the experiment, where the two fluorescent markers are not colocalized. In addition, the cumulative pixel correlation plots may be used to choose adequate threshold value, which would separate background fluorescence from the signal. Alternatively, one may also use automatic techniques to determine the threshold value [8]. The 'graythresh' function in Matlab Image Processing Toolbox (Mathworks, Natick, MA), chooses the threshold to minimize the intraclass variance of the selected and non-selected pixels [9].

If the pixels with mid range intensities are not colocalized due to unspecific staining of background structures or due to the channel cross-talk, one may also evaluate colocalization using correlation technique. Pearson's r correlation coefficient for fluorescence intensity values is calculated together with P -values for testing the hypothesis of no correlation. Each P -value is the probability of getting a correlation as large as the observed value by random chance, when the true correlation is zero. Since the number of pixels in one image is very high (tens of thousands), the correlation value is statistically significant even at a low value. For this reason, the analysis of a single or a few images is not sufficient. Instead, we present here a convenient and fast approach, which enables the analysis of many images, quantifies the colocalization levels of the two markers for each cell image and enables to compare these values with those from control experiments. Moreover, using our automated approach one can also implement the colocalization

analysis described by Manders et al. [10], where the two coefficients represent the fraction of colocalized objects of each component of a dual-channel image. Automation of analyses eliminates the introduction of biases due to the image adjustments performed by the user and provides the means for fast and precise analyses of large groups of images.

Acknowledgements

We thank Professors Anna-Liisa Nieminen (At Case Western Reserve University, Cleveland, USA) and Tom Rapoport (Harvard Medical School, Boston, USA) for kindly donating the plasmids encoding fluorescent fusion proteins Cytc-GFP and Sec61-GFP, respectively. This work was supported by the Ministry of Education, Sciences and Sports of the Republic of Slovenia fund P3 521 0381, the R01 NS36665-05 grant and the European Community Grant QLG3-CT 2001-2004.

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