Dynamics of Cellular Responses studied by Quantitative Colocalization Analysis

Olga Zinchuk[1] and Vadim Zinchuk[2] 1. Department of Ophthalmology, and 2. Department of Anatomy and Cell Biology, Kochi University Faculty of Medicine, Nankoku, Kochi, Japan

BIOGRAPHY

Dr Olga Zinchuk obtained an MD in ophthalmology from Ukrainian State Medical University, Kiev, Ukraine, in 2000. She then worked as a physician ophthalmologist in Kiev. Since 2003 Olga has been in the graduate school in the Department of Ophthalmology, Faculty of Medicine, Kochi University, Kochi, Japan. Her research interests include the mechanisms of signaling via G-protein-coupled receptors and the use of advanced computational techniques for image analysis.

ABSTRACT

Quantitative colocalization analysis is a tool to objectively estimate the degree of overlap (colocalization) of fluorescently labelled antigens in biological microscopy. It allows the drawing of unbiased conclusions about their interaction and function. This article summarizes the theoretical basis of quantitative colocalization analysis and describes the use of the technique. The applicability and correct interpretation of the results of colocalization coefficients calculations is given for the investigation of the role of platelet activating factor (PAF) and its receptor (PAF-R) in the recruitment of eosinophils into the conjunctiva. Quantitative colocalization analysis helped to determine that the recruitment of eosinophils was accomplished by direct action of PAF on the PAF-R and that the interaction of both PAF-R and major basic protein antigens with PAF was required for eosinophil influx.

KEYWORDS

confocal microscopy, multicolour fluorescence, quantitative colocalization analysis, colocalization software, platelet activating factor receptor, eosinophils

AUTHOR DETAILS

Dr Olga Zinchuk,
Department of Ophthalmology,
Kochi University Faculty of Medicine,
Kohasu, Okoh-cho, Nankoku,
Kochi 783-8505, Japan
Tel: +81 88 880 2391
Email: olga@med.kochi-u.ac.jp

Microscopy and Analysis 20(5):S13-S15 (UK), 2006

INTRODUCTION

The observation of colocalized antigens has become one of the most widely noticed and highly credited findings in modern cell and molecular biological studies. Although colocalization is relative, information on the appearance of distinct molecules at the same physical location can be of particular importance. The phenomenon of colocalization can be of even greater biological significance when evaluated quantitatively. We have pioneered the wider use of quantitative colocalization analysis for a better understanding of the results of cell and molecular biological experiments.

Colocalization in fluorescence microscopy is defined as the presence of two or more types of fluorescent molecules at the same location. Physically, this means that the colours emitted by them occupy the same pixel in the image. Biologically, this means that two or more different molecules attach to the same structure in the cell. Quantitative colocalization provides an answer to the question: "Does protein A overlap/colocalize with protein B in a given image, and if so to what degree?"

Although the number of reports employing quantitative assessment is growing steadily [1-5], in the majority of publications colocalization is still described in vague terms, such as "full", "partial", or "incomplete". Such evaluations are usually judged by the naked eye only and are too superficial to fully understand the details of the underlying biological processes. Most importantly, they do not allow the following of the changes of the degree of colocalization in a timely fashion, which might have been applicable to the antigens under study, but were not determined because quantification was not used.

In this article, we evaluate colocalization quantitatively by studying the dynamic changes of the expression of platelet activating factor receptor (PAF-R) and major basic protein (MBP) antigens in the conjunctiva. MBP, a marker of eosinophils, is an important player in immediate hypersensitivity reactions and in allergic conjunctivitis. Platelet activating factor (PAF) is one of the most potent chemotactic agents for the recruitment of eosinophils. The dynamics of PAF-R expression by eosinophils in the conjunctiva is important for developing therapeutic strategies for the treatment of allergic conjunctivitis.

MATERIALS AND METHODS

1% PAF C18 (1-O-octadecyl-2-acetyl-sn-glycero-3-phosphocholine, from Cayman Chemical) was applied topically in eye drops to Brown Norway rats. The eyes were enucleated with attached lids and intact conjunctiva at 30 min, or 2, 6, and 24 h following PAF instillation. In controls, PBS was used instead of PAF solution. Samples were examined using routine histology, immunohistochemistry, confocal immunofluorescence microscopy, quantitative colocalization analysis and RT-PCR.

Sections for immunohistochemistry and confocal immunofluorescence microscopy were incubated with anti-PAF-receptor (Cayman Chemical) and anti-major basic protein (MBP; Biodesign International) primary antibodies. Sections for immunohistochemistry were incubated with appropriate biotinylated secondary antibodies. Immunostaining was visualized using ABC (Vector Labs) and DAB (Sigma) kits. Immunopositive cells were counted throughout the sections.

Sections for confocal immunofluorescence microscopy were exposed to the corresponding secondary antibodies (conjugated with Alexa 488 and Alexa 594; Molecular Probes). In controls, primary antibodies were omitted from the labelling process. Sections were examined using a Zeiss Axiovert 135M microscope equipped with a Zeiss LSM 410 confocal laser scanning system. Double fluorescence for green and red channels was imaged using

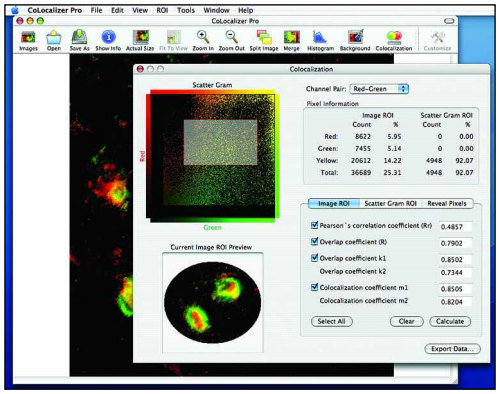

Figure 1:
Main and colocalization windows of computer software, CoLocalizer Pro, used to quantify colocalization. The software allows one to calculate all known colocalization coefficients on the selected region of interest, to analyze scattergram and scattergram ROIs, and to reveal colocalized or selected pixels. All calculations can be performed without the need to be connected to a confocal microscope.

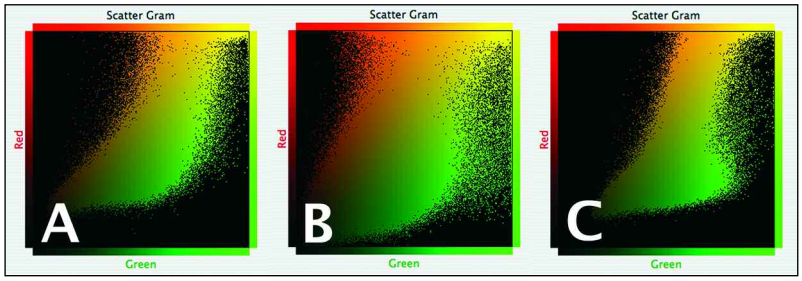

Figure 2:
Different scattergrams revealing pixel distribution in analyzed images (A-C). Note differences of distribution of colocalized pixels along the diagonal of each scattergram. The scattergram in C shows the least number of pixels along the diagonal and thus the lowest values of colocalization.

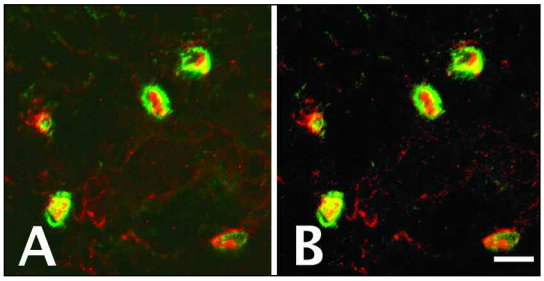

Figure 3:
Images before (A) and after (B) the background correction. The background corrected image looks different as unwanted background pixels were removed. Scale bar = 20 µm.

excitation of an argon-krypton-neon laser at wave lengths of 488 and 543 nm. Double stained images were obtained by sequential scanning for each channel to eliminate the crosstalk of chromophores and to ensure the reliable quantification of colocalization.

Confocal images were transferred to a Macintosh Dual PowerPC G5 for analysis. Colocalization of antigens was evaluated quantitatively using CoLocalizer Pro software (CoLocalization Research).

Colocalization analysis takes into consideration a pair of images from two different channels and then generates a single image in which common events within a selected range will be counted. It relies on a number of coefficients for estimating the degree of colocalization as well as enabling the viewing of actual areas of colocalization on the images.

Several algorithms, such as Pearson's correlation coefficient, overlap coefficient according to Manders, overlap coefficients k1 and k2, colocalization coefficients m1 and m2, and colocalization coefficients M1 and M2 [6-9] were developed by which colocalization can be evaluated quantitatively. These coefficients use different approaches to estimate colocalization and have different sensitivity and applicability. A description of the coefficients is summarized in the Table.

Pearson's correlation coefficient (PCC), Manders overlap coefficient (MOC) [9], and overlap coefficients k1 and k2 were employed to evaluate colocalization. Calculations were performed on the whole image serving as a region of interest (ROI) with the help of the professional scientific software CoLocalizer Pro (Figure 1).

In addition to coefficients, other important information about the analyzed image can be obtained by evaluating its scattergram (Figure 2) which estimates the amount of each detected antigen based on the colocalization of PAF-R (red, y axis) and MBP (green, x axis). Colocalized pixels of yellow colour are located along the diagonal of each scattergram.

It is important to remember that for quantitative colocalization analysis images should have their background corrected by removing pixels of unwanted levels. The extent of background correction depends on a variety of factors, including the intensity of immunofluorescence and the models of microscopes used to acquire images. This step is highly critical and will impact the outcome of coefficient calculations. In our experiment the background was corrected using the threshold value for all channels to remove background and noise levels completely (Figure 3).

Statistical comparisons of the number of infiltrating cells and the degrees of colocalization were performed using the Mann-Whitney U test. A $p<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Confocal immunofluorescence microscopy showed the expression of PAF-R on eosinophils at all time points, including controls (Figures 4 A-E), indicating the presence of PAF-R on eosinophils even in the intact state (Figure 4A).

Quantitative colocalization analysis demonstrated a high degree of colocalization even in controls. It showed a gradual increase as the PAF-induced inflammation developed (Figure 5A). When the number of infiltrating eosinophils started to decrease (by 24 h after PAF administration) (Figure 5B), the number of PAF-R positive cells (Figure 5C) and the degree of colocalization still kept increasing. These findings might indicate that eosinophils found in the conjunctiva sections at this time point maintained an elevated expression of PAF-R. On the other hand, they might reflect the changes of the expression of PAF-R antigen by other cells in the conjunctiva.

Calculation of two more colocalization coefficients, k1 and k2, has helped to shed light on these questions. The degree of PAF-R expression gradually increased during our experiment (Figure 5D) while the expression of MBP antigen decreased. The peak number of infiltrating eosinophils coincided with the moment, at 6 hours after PAF administration, when k1 equalled k2, i.e. the intensities of MBP and PAF-R antigen signals were the same. After this period of time, the k1 continued to grow, but the number of infiltrating eosinophils was no longer increasing. This suggests that the interaction of both PAF-R and MBP antigens with PAF is required for the recruitment of eosinophils.

mRNA expression in all samples remained approximately constant in all examined samples and at all time points, showing signs of neither up- nor downregulation (data not presented). This result suggests that these chemokines are not involved in the process of eosinophil recruitment into the conjunctiva following PAF challenge.

In summary, quantitative colocalization analysis in combination with cell counting and RT-PCR led to the conclusions that: 1. administration of PAF caused an increase of the degree of PAF-R expression by eosinophils in the course of PAF-induced conjunctivitis; 2. the interaction of both PAF-R and MBP antigens with PAF is required for the recruitment of eosinophils; and 3. the recruitment of eosinophils in PAF-induced conjunctivitis is accomplished via the direct action of PAF on the PAF-R. Finally, the fact that we were able to quantify the degree of colocalization of MBP and PAF-R antigens and the dynamic changes in the degree of PAF-R expression, allowed us to determine the proper time frame for the use of PAF-R antagonists in clinical settings. These conclusions would have been impossible to make without the quantification of colocalization.

Table: Characteristics of coefficients used for colocalization estimation.

Coefficients and Meaning	Values	Use
Pearson's correlation coefficient: One of the standard measures in pattern recognition. Describes the correlation of the intensity distribution between channels. Considers only similarity between shapes, while ignoring the intensities of signals.	-1.0 to 1.0, where -1.0 indicates no any overlap and 1.0 is a perfect correlation.	Can be used in any colocalization experiment.
Overlap coefficient according to Manders: Indicates an actual overlap of the signals. Represents the true degree of colocalization. This coefficient is insensitive to the limitations of typical fluorescence imaging, such as efficiency of hybridization, sample photobleaching, and camera quantum efficiency.	0 to 1.0. 0.7 implies that 70% of both selected channels colocalize.	Can be used in any colocalization experiment. Especially applicable when fluorescence of one antigen is stronger than the other.
Overlap coefficients k1 and k2: Split the value of colocalization into two separate parameters allowing the determination of the contribution of each antigen to the areas with colocalization.	Variable.	Can be used in any colocalization experiment.
Colocalization coefficients m1 and m2: Describe contribution of each one from two selected channels to the pixels of interest.	0 to 1.0. m1 and m2 of 1.0 and 0.3 for red-green pair imply that all red pixels colocalize with green, but only 30% of green pixels colocalize with red.	Can be used in any colocalization experiment.
Colocalization coefficients M1 and M2 Identical to m1 and m2, but applied to analyze scattergram ROI.	Identical to m1 and m2.	Identical to m1 and m2.

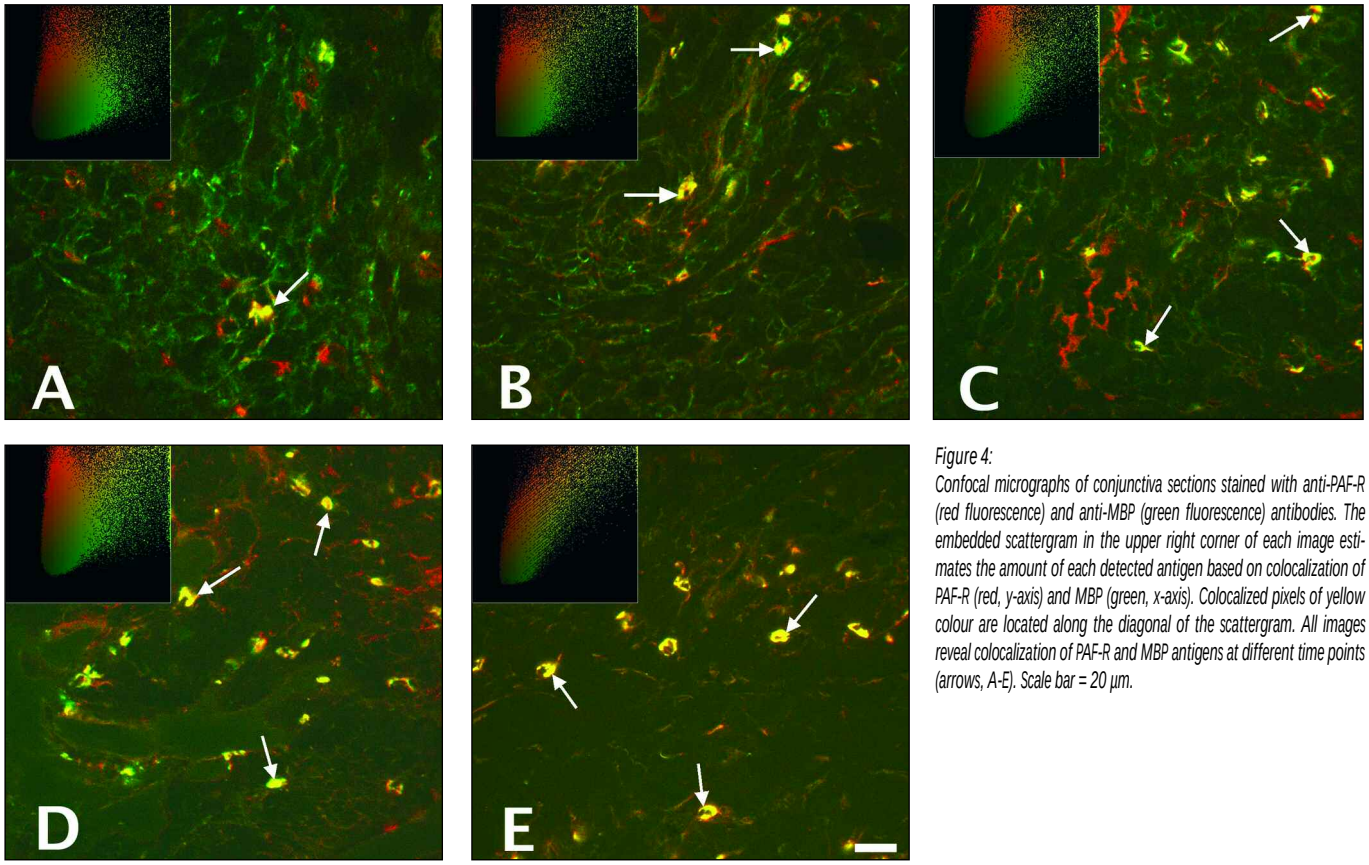

Figure 4:
Confocal micrographs of conjunctiva sections stained with anti-PAF-R (red fluorescence) and anti-MBP (green fluorescence) antibodies. The embedded scattergram in the upper right corner of each image estimates the amount of each detected antigen based on colocalization of PAF-R (red, y-axis) and MBP (green, x-axis). Colocalized pixels of yellow colour are located along the diagonal of the scattergram. All images reveal colocalization of PAF-R and MBP antigens at different time points (arrows, A-E). Scale bar = 20 µm.

CONCLUSIONS

This article has reviewed the use of quantitative colocalization analysis in biomedical experiments. We briefly described the theoretical basis of the technique of quantitative colocalization analysis and gave an example of how it can be used and how the results of coefficient calculations can be interpreted. Quantitative colocalization allows the objective comparison of antigen colocalization and the tracking of dynamic changes in colocalization. It also allows the measurement of the contribution of each particular antigen to the process of colocalization and a comparison of the extent of this contribution. Importantly, the latest progress in the field, such as use of computer-assisted analysis, makes quantitative colocalization analysis a highly reliable and reproducible technique. The use of quantitative colocalization analysis is thus fully justified in any double-immunofluorescence confocal experiment which finds colocalization of antigens as it can provide important additional details about the physiological processes under study.

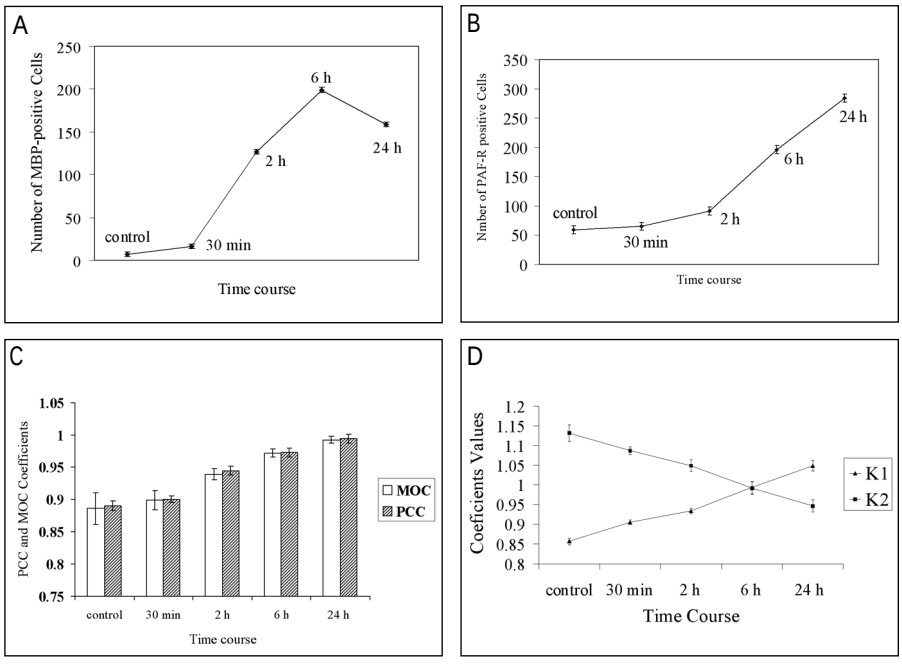

Figure 5:
Dynamic changes of the number of PAF-R and MBP positive cells and colocalization parameters. Quantitative representation of the changes of the number of PAF-R and MBP immunopositive cells (A and B), MOC and PCC coefficients (C), and overlap coefficients K1 and K2 (D) in the dynamics of PAF-induced conjunctivitis.

REFERENCES

1. *Criscuoli, M. L.. et al. Tumor metastasis but not tumor growth is dependent on Src-mediated vascular permeability. Blood 105:1508-1514, 2005.*
2. *Kato, T. et al. Atrial natriuretic peptide promotes cardiomyocyte survival by cGMP-dependent nuclear accumulation of zyxin and Akt. J Clin. Invest. 115:2716-2730, 2005.*
3. *Manders, E. M. et al. Dynamics of three-dimensional replication patterns during the S-phase, analysed by double labelling of DNA and confocal microscopy. J. Cell Science 103(3):857-862, 1992.*
4. *Petrescu, A. D. et al. Physical and functional interaction of Acyl-CoA-binding protein with hepatocyte nuclear factor-4 alpha. J. Biol. Chem. 278:51813-51824, 2003.*
5. *Zinchuk, O. et al. Dynamics of PAF-induced conjunctivitis reveals differential expression of PAF receptor by macrophages and eosinophils in the rat. Cell Tissue Research 317:265-277, 2004.*
6. *Zinchuk, V. et al. Experimental LPS-induced cholestasis alters subcellular distribution and affects colocalization of Mrp2 and Bsep proteins: a quantitative colocalization study. Microscopy Research Technique 67:65-70, 2005.*
7. *Zinchuk, O. et al. Direct action of platelet activating factor (PAF) induces eosinophil accumulation and enhances expression of PAF receptors in conjunctivitis. Mol. Vis. 11:114-123, 2005.*
8. *Agnati, L. F. et al. New methods to evaluate colocalization of fluorophores in immunocytochemical preparations as exemplified by a study on A2A and D2 receptors in Chinese hamster ovary cells. J. Histo. Cytochem. 53:941-953, 2005.*
9. *Manders, E. M. M. et al. Measurement of co-localization of objects in dual-colour confocal images. Journal of Microscopy 169:375-382, 1993*