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A THREE DIMENSIONAL QUANTITATIVE APPROACH FOR COLOCALIZATION STUDY OF CYCLIN A AND P34CDK1 BY CONFOCAL MICROSCOPY

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ABSTRACT

In mammalian cells, cyclin A and cyclin B are known to regulate P34cdk1 activity during the entry into mitosis, whereas cyclin A also regulates p33cdk2 kinase. Confocal laser scanning microscopy acquisition and three dimensional image analysis were performed to quantitatively estimate cyclin A and P34cdk1 colocalization on lymphoid RAMOS cell line. Cytofluorograms were used to derive parameters estimating colocalization. Colocalization globally increased when cells accumulated cyclin A. However, results demonstrated that some positive voxels were not colocalized.

Key words : colocalization, confocal microscopy, image analysis, double labelling, cyclin A, P34cdk1.

INTRODUCTION

We propose to study cyclin A and P34cdk1 colocalization by means of confocal laser scanning microscopy (Pawley, 1995) and three dimensional image analysis (Meyer, 1992).

P34cdk1 is a protein kinase involved in mitosis events regulated by two cyclins: cyclin A and cyclin B (Lewin, 1990). Cyclin A is also known to regulate another kinase, p33cdk2. Furthermore, cyclin A content changes during the cell cycle, it accumulates in the nucleus and is destroyed during mitosis; whereas P34cdk1 is present in both cytoplasm and nucleus of cycling cells. This partial relationship between cyclin A and P34cdk1 needs to be studied by a colocalization approach.

Confocal laser scanning microscopy provides thin optical sections through three-dimensional biological specimens and availability of multiwavelength configurations make it worthy for colocalization studies of cells with respect to their three dimensional integrity.

MATERIAL AND METHODS

Biological material

Cells were obtained from a RAMOS cell line (issued from a Burkitt's lymphoma). All preparation steps were carried out at room temperature. After elimination of aggregated cells by Ficoll-Hypaque separation (d=1.077, 380g for 20 minutes) and washing in phosphate buffer saline (PBS), cells were laid on adhesion slides (Biorad, Munich, Germany) for 15 minutes. Cells were fixed in paraformaldehyde 0.5% for 30 minutes followed by methanol for 5 minutes. Before antibody labelling, cells were incubated for 15 minutes in bovine serum albumine 3%. Each antibody was sequentially added on each reaction field after PBS washing. Cells were successively incubated with mouse IgE anti-cyclin A antibody (Pharmingen, San Diego, CA) at a 1:20 dilution for 1 hour, with rat anti-mouse IgE biotin (Pharmingen) and rabbit anti-rat Ig biotin (Dakopatts, Copenhagen, Denmark), both at a dilution of 1:100 for 30 minutes, and with streptavidin-cyanine 3 conjugate (Jackson, West Groove, PA) at a dilution of 1:400 for 30 minutes. Cells were then post-fixed in 0.5% paraformaldehyde for 10 minutes. For P34cdk1 immunofluorescence, mouse IgG anti-P34 antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) was added at a dilution of 1:20 for 1 hour and successively revealed by fluorescein isothiocyanate (FITC) goat anti-mouse IgG (Caltag, San Francisco, CA) and FITC swine anti-goat (Coulter, Miami, FL), both at a dilution of 1:50 for 30 minutes. Negative controls without the first antibody (anti-cyclin A or anti P34cdk1 antibody) were realized. After washing in PBS, cells were mounted in buffered glycerol (pH 8.5), coverslipped and sealed with clear nailpolish.

Image acquisition

Cells were acquired on an LSM 10 confocal laser scanning microscope (Zeiss, Oberkochen, Germany) through a 40X oil immersion objective with a 1.3 numerical aperture. Cyanine 3 was excited by a 543 nm wavelength Helium-Neon laser and its fluorescent emission light was detected through a 590 nm long-pass filter. FITC was excited by a 488 nm wavelength Argon laser and detected through a 515-545 band-pass filter. Colours of the fluorescent emission reaching the photodetector respectively corresponded to red and green colours. Each image was averaged 16 times to enhance the signal to noise ratio. A total of 80 images (40 for each colour) spaced out 0.5 µm apart were acquired to reach a final voxel resolution of 0.1x0.1x0.48 μm^3 after refractive index correction (Hell et al. 1993).
Images were further treated and analysed on an Iris Indigo R4000 XS24 workstation (Silicon Graphics Inc., Mountain View, CA) with Visilog 4.1.4 software package (Noesis SA, Vélizy-Villacoublay, France) using our own three dimensional image analysis functions written in C.

Image preprocessing

Reliability of colocalization measurement is influenced by background, Poisson noise and cross-talk (Manders 1992). In order to attenuate background variation and Poisson noise, the following preprocessing were carried out. Images, previously averaged during acquisition, were also smoothed with a 3x3x3 gaussian low pass filter in order to suppress almost all noise. Moreover, the modal value of the background was estimated and subtracted from the original image, negative values being set to zero. Due to the double laser excitation system and the specific emission filter set, cross-talk between fluorochromes, as analysed on negative controls, was negligible (Mossberg and Ericsson, 1990).

Afterwards, a threshold was applied on the images of cyclin A labelling to extract the cell shape from the background. Each segmented cell image was then cut and saved in a single file. All further measurements were made only in voxels belonging to the cell.

Cyclin A and P34cdk1 contents

Before dealing with colocalization, we measured the integrated grey level for each labelling and in each cell. These measurements corresponded to cyclin A (Q_{CA}) and P34cdk1 (Q_{P34}) contents.

Dual margination

To measure the different nuclear and cytoplasmic intracellular localization of both cyclin A and P34cdk1 distribution, we used the physics concept of inertia (Delorme et al., 1994). By computing the ratio of cyclin A inertia over P34cdk1 inertia ($marg_{CA/P34}$) we obtained a value indicating which of cyclin A or P34cdk1 is the most central: cyclin A ($marg_{CA/P34}<1$) or P34cdk1 ($marg_{CA/P34}>1$).

Colocalization

The colocalization measurements were made on dual colour contour plots (cytofluorograms). To assess the degree of colocalization , we measured colocalization ratio as the ratio of voxels over two fixed thresholds (Taneja, 1992; Hassan, 1994; Demandolx, 1995). The threshold values were chosen in order to well separate the background. The colocalization percentage ($c\%$) was obtained as the number of colocalized voxels divided by the total number of positive voxels (red + green + colocalized voxels). From the same data, we computed the percentage of red ($\%R$) and green voxels ($\%G$).

We also measured the Pearson's correlation coefficient (r) (Manders, 1993) and the slope of the correlation line (b). R and b reflect the colocalization degree; but b also takes into account the relative intensity of both labelling.

To obtain more complete features, we computed the red and green mean values for the 25% brightest red voxels (G_R, R_R) and for the 25% brightest green voxels (G_G, R_G). The ratios of the green means ($Gr=G_G/G_R$) and of the red means ($Rr=R_R/R_G$) were then evaluated. Values close to 1.0 correspond to high colocalization. Alternatively, a value of Gr (respectively Rr) much over 1.0 means that low green (red) values are associated to high red (green) values.

RESULTS

Cyclin A and P34cdk1 contents

According to cyclin A content (Fig. 1), most of the cells showed a weak, negative staining. In the rest of the cell population, cyclin A gradually increased. P34cdk1 (Fig. 1) showed slightly different behaviour. One part of the cell population was weakly stained; a second part showed a brighter but constant staining; whereas few other cells contained higher P34cdk1 content. Significant correlation between cyclin A and P34cdk1 was observed (r=0.88).

Dual margination

Margination ratio $marg_{CA/P34}$ diminished while cyclin A increased in the cell, to reach values less than 1.0.

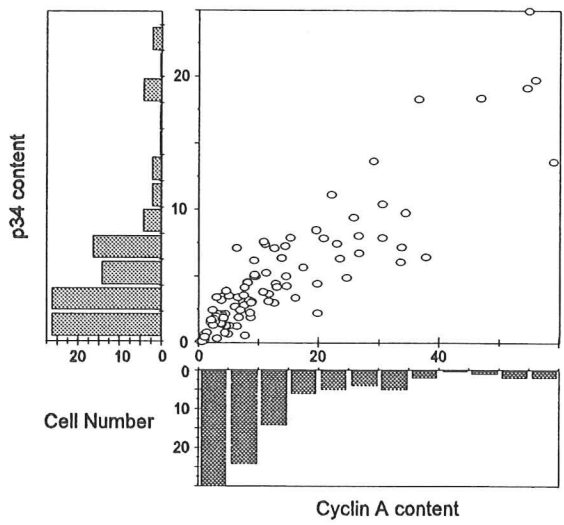

Fig. 1. Cell distribution according to cyclin A and P34cdk1 contents.

Colocalization

Cytofluorograms (Fig. 2) are similar to Fig. 1 but for a single cell instead of the full population. According to cyclin A content, some colocalization (*c%, r*) parameters increased to reach a constant level. On the other hand, other parameters decreased (*b, Rr, Gr)*.

DISCUSSION

Cyclin A content variation may be explained by its periodic accumulation behaviour (Pines and Hunter, 1991). From P34cdk1 results, three kinds of cells may exist: quiescent cells with poor P34cdk1 and cyclin A contents, cycling cells with higher P34cdk1 content and variable cyclin A content and a contingency of cells exhibiting high cyclin A and P34cdk1 contents. It is possible that P34cdk1 content increased during the cell cycle. Nevertheless, due to the pathological origin of the RAMOS cell line, some abnormal cells may overexpress P34cdk1 and cyclin A. Cyclin A and P34cdk1 contents gave only a global view without any knowledge about their intracellular distribution inside each cell.

Dual margination values indicated that cyclin A distribution was more nuclear than P34cdk1. This was in agreement with our visual observation where cyclin A labelling appeared stronger inside the nucleus, though P34cdk1 labelling still remained bright inside the cytoplasm.

Colocalization results may be deduced from a case where cyclin A accumulated in the nucleus and P34cdk1 remained present in both cytoplasm and nucleus; i.e. cyclin A and P34cdk1 colocalized mainly in the nucleus. Colocalization variables r and %c indicated that colocalization globally increased when the cell accumulated cyclin A. Their high values which suggested an important colocalization of the two proteins, may be explained by the high nuclear to cytoplasmic ratio of RAMOS cells. Nevertheless, other variables such as *Rr* or *Gr* demonstrate that cyclin A and P34cdk1 were partly colocalized in the cell.

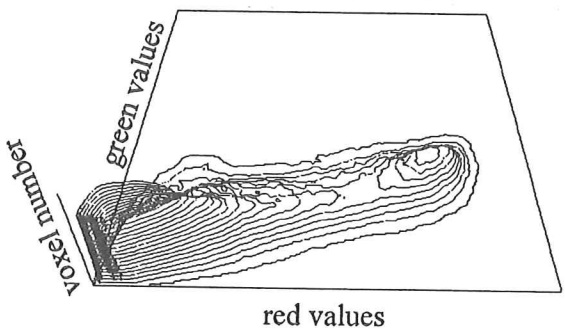

Fig. 2. Dual colour contour plot (cytoflurogram). Contour lines represent, inside a single cell, the number of voxels with each couple of red-green colour.

CONCLUSION

This paper shows that several colocalization parameters are necessary to describe unequivoquely P34cdk1 and cyclin A colocalization. Colocalization still remains an open field and other methods might better summarize cytofluorograms.

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REFERENCES

Delorme R, Souchier C, Ffrench M, Bryon PA. Confocal image analysis of three dimensional intracellular protein distribution in lymphoid cells. Bioimaging 1994; 2: 69-77.

Demandolx D, Davoust J. Multicolor analysis in confocal immunofluorescence microscopy. J Trace Microprobes Tech 1995; in press.

Hassan B, Errington RJ, White NS, Jackson DA and Cook PR. Replication and transcription sites are colocalized in human cells. J Cell Sci 1994; 107: 425-34.

Hell S, Reiner G, Cremer C , Stelzer EHK. Aberrations in confocal fluorescence microscopy induced by mismatches in refractive index. J Microsc 1993; 169: 391-405.

Lewin B. Driving the cell cycle : M phase kinase, its partners ans substrates. Cell 1990; 61: 743-52.

Manders EMM, Verbeek FJ, Aten JA. Measurement of co-localization of objects in dual colour confocal images. J Microsc 1993; 169: 375-82.

Meyer F. Mathematical morphology from two to three dimensions J. Microsc 1992; 165: 5-28.

Mossberg K, Ericsson M. Detection of doubly stained fluorescent specimens using confocal microscopy. J Microsc 1990; 158: 215-24.

Pawley JB. Handbook of biological confocal microscopy. New York: Plenum Press, 1995: 1-232.

Pines and Hunter. Human cyclins A and B1 are differently located in the cell cycle-dependent nuclear transport. J Cell Biol 1991; 115: 1-17.

Taneja K, Lifhitz LM, Fay F, Singer R. Poly(A) RNA Codistribution with microfilaments: evaluation by in situ hybridization and quantitative digital imaging microscopy. J Cell Biol 1992; 5: 1245-60.

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