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# A simple technique of image analysis for specific nuclear immunolocalization of proteins

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**Summary**

Colocalization of fluorescent signals in confocal microscopy is usually evaluated by inspecting merged images from different colour channels or by using commercially available software packages. We describe in this paper a simple method for assessment of nuclear localization of proteins in tissue sections through confocal immunolocalization, propidium iodide counterstaining and image analysis. Through a macro command developed for the public domain, Java-based software IMAGEJ, red, green, blue (RGB) images are automatically split in the red and green channels and a new image composed of the nonblack pixels coincident in both channels is created and inverted for better visualization. This method renders images devoid of both, extranuclear staining and background, thus emphasizing the nuclear signal. The resulting images can easily be used for comparison or quantification of the results. Given the simplicity of the technique and the worldwide diffusion of the software utilized, we think that this method could be useful in order to define standards of colocalization in confocal microscopy.

## Introduction

Confocal microscopy allows for easy immunolocalization of antigens in tissues and cells, even at the subcellular level. Thus, it is possible to check, in a confocal plane, if a protein of interest is located in the cell surface, into the cytoplasm or within the cell nucleus. This is especially important in the case of transcription factors whose activity can be regulated by mechanisms of nuclear translocation and/or nuclear export (Pemberton & Pascal, 2005). The presence or absence of these factors in the nucleus is functionally relevant.

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For example, $\beta$-catenin is a protein which plays a dual role in cells depending on its precise localization. $\beta$-catenin can remain associated with cadherins at the intracellular side of the membrane, thus contributing to cell adhesion. When $\beta$-catenin levels increase in the cytoplasm, or if intercellular junctions become disorganized, it can translocate within the cell nucleus and activate transcription associated to Lef/Tcf factor. Thus, nuclear content of $\beta$-catenin is relevant in order to characterize cell behaviour and function (Brembeck *et al.*, 2006). Another factor whose cellular localization is essential for its function is Snail, a zinc-finger transcription factor involved in epithelial-mesenchymal transitions (Nieto, 2002). Snail function as transcription factor is modulated by a mechanism of GSK3$\beta$-dependent phosphorylation, nuclear export and degradation (Domínguez *et al.*, 2003; Peinado *et al.*, 2005).

Nuclear localization of proteins in tissue sections through immunocytochemistry and confocal microscopy is usually performed by direct inspection of the signal obtained overlapped with a standard nuclear label (DAPI, Hoescht blue or propidium iodide). On the other hand, there are commercially available image analysis software that allow to emphasize and quantify colocalization, usually by using a third colour where the fluorochromes used are superimposed. In the first case, protein colocalization can be difficult to assess, especially when signal is weak and/or background is strong. On the other hand, image analysis software can be expensive, complex and it is usually designed just for a particular operative system. Thus, although some authors have recently described good methods to analyze colocalization (Kreft *et al.*, 2004; Goucher *et al.*, 2005; Jaskolski *et al.*, 2005), a simple, standard method to underscore signal colocalization has not been widely accepted.

We have developed a simple, rapid and cost-free method to obtain images showing exclusively nuclear localization of proteins by using a public domain, Java-based software. The images obtained are devoid of background (even in the

cases in which the strong background was generated by secondary antimouse IgG antibodies on mouse tissue). The aim of this paper is to describe this technique and to show their possibilities. With this purpose we have designed three experiments in order to show nuclear localization of the above quoted factors, $\beta$-catenin and Snail, and colocalization of another transcription factor, HNF1, with the proliferating-cell nuclear antigen PCNA.

## Material and methods

### *Immunohistochemistry and confocal microscopy*

We have performed standard immunocytochemistry on paraffin sections of mouse and chick embryos. Deparaffined sections were blocked with Avidin-Biotin blocking reagent (Vector, Burlingame, CA) and nonspecific binding sites were saturated with 16% sheep serum, 1% bovine serum albumin and 0.5% Triton X-100 in Tris-PBS (SBT). Then, the sections were incubated with the primary antibody overnight at 4°C diluted in SBT. After three washes in Tris-PBS (5 min) the slides were incubated in antirabbit or antimouse IgG biotinylated antibody (Sigma, 1 : 100 in SBT, 1 h), washed again and incubated in Extravidin-FITC (Sigma, 1 : 150 in Tris-PBS, 1 h) (Sigma, Madrid, Spain). Between the first and second wash the sections were incubated in propidium iodide dissolved in PBS (5 $\mu$g/mL, 10 s). After the final wash, the sections were mounted in a 1 : 1 PBS/glycerol solution and analyzed using a Leica TCS-NT laser confocal microscope. Localization of the Snail protein required of tyramide amplification of the signal according to the supplier's instructions (TSA amplification kit, Perkin-Elmer, Madrid, Spain).

Double immunocytochemistry was performed by incubating the sections overnight with both, monoclonal and polyclonal antibodies, and then with both, an antirabbit IgG biotinylated antibody (1 : 100) and a TRITC-conjugated antimouse IgG antibody (1 : 100). After washing the sections were finally incubated in Extravidin-FITC, washed and mounted as above described.

Rabbit polyclonal anti-HNF1 (sc-8986) was obtained from Santa Cruz and used at 1 : 100 dilution. Mouse monoclonal anti-$\beta$-catenin was from BD Transduction (C19220) and used at 1 : 100 dilution. Monoclonal mouse anti PCNA (Sigma) was used at a 1 : 100 dilution. The mouse monoclonal anti-Snail antibody, a kind gift from Antonio García de Herreros, (Pompeu Fabra University, Barcelona), was used at 1 : 25 dilution.

### *Image analysis*

We performed image analysis using the public domain, free software IMAGEJ (Image processing and analysis in Java, http://rsb.info.nih.gov/ij/), a Java application which runs in most operative systems. Confocal images (TIFF or JPG formats) were first split in red, green and blue channels using the RGB split command. Blue channel was discarded and then we performed an operation with the red and green images using the application 'image calculator' with the option 'AND'. In this way we obtained an image constituted of all the nonblack (i.e. non-null) pixels present in both channels at the same time. We finally inverted the images to get different levels of grey on a white background.

This is a macro command which automates the process:

```
run('RGB Split');

close();

selectImage(1);

run('Rename...', 'title=green');

selectImage(2);

run('Rename...', 'title=red');

run('Image Calculator...', 'image1=[green] operation=AND image2=[red] create');

run('Invert');

run('Rename...', 'title=Result');

selectWindow('red');

run('Close');

selectWindow('green');

run('Close');
```

We assume that these levels of grey, when used on propidium iodide counterstained images, represent an assessment of the nuclear localization of the protein immunostained with fluorescein. We have used also this method to colocalize two nuclear proteins, the proliferation marker PCNA and the hepatoblast marker HNF1, allowing for specific assessment of proliferation of the hepatoblasts in the developing mouse liver.

## Results and discussion

### *$\beta$-catenin in chick endocardial cushions*

We have used our technique to study nuclear $\beta$-catenin levels in the endocardium and endocardial cushion mesenchyme of the developing chick heart. We know that epithelial-mesenchymal transition of the endocardium leads to formation of valvuloseptal mesenchyme and this process involves nuclear translocation of $\beta$-catenin (Hurlstone *et al.*, 2003; Liebner *et al.*, 2004).

The image obtained with our technique (Fig. 1D) clearly shows this nuclear accumulation of $\beta$-catenin, especially in the endocardial areas of epithelial-mesenchymal transition (arrows), and it also shows a definite gradient of accumulation

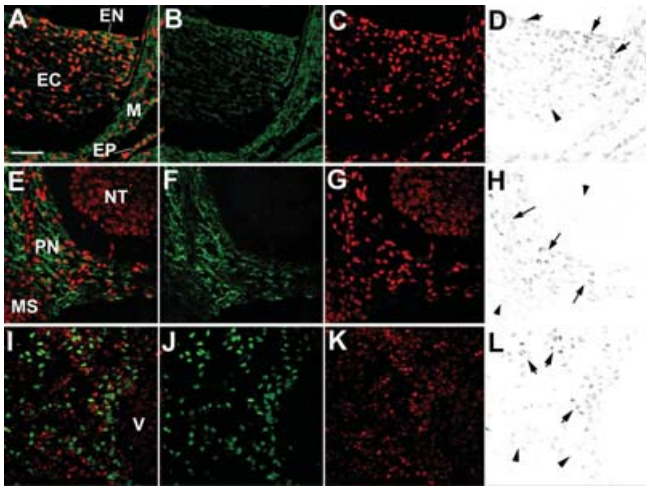


**Fig. 1.** Results after our technique of image analysis for nuclear localization of proteins. The first column shows confocal images, the second and the third columns show the green and red channels and the fourth column shows images obtained with our technique, where the nonblack pixels coincident in the green and red channels are shown in grey scale. **A–D:** Colocalization of $\beta$-catenin (green) with propidium iodide (red) in the atrioventricular endocardial cushion (EC) of a chick embryo. In the image obtained with our technique, a gradient of nuclear content of $\beta$-catenin from the endocardial and subendocardial areas (arrows) to the innermost part of the cushion (arrowhead) becomes evident. This feature is much more difficult to appreciate in the original confocal figure or in the green channel, due to the signal from the extranuclear $\beta$-catenin. EN: endocardium, EP: epicardium, M: myocardium. **E–H:** Colocalization of the transcription factor Snail (green) with propidium iodide (red) in the perineural plexus (PN) of a mouse embryo. The antimouse IgG secondary antibody caused a strong background which was completely eliminated by our technique. Note the specific nuclear staining in the perineural mesenchyme (arrows) but not in the neural tissue (NT) or in other mesenchymal cells (MS). **I–L:** Colocalization of the hepatoblast marker HNF1 (green) with the proliferation marker PCNA (red) in the liver of a mouse embryo. Clusters of proliferating hepatoblasts are revealed in L (arrows), whereas other hepatoblasts are not double labelled (arrowheads). This feature is not apparent in the original figures, where proliferating cells can be of different types. V: vein. Scale bar for all figures = 50 m.

between the endocardial/subendocardial cells and the inner cells of the cushions (arrowhead), suggesting a decrease in the amount of nuclear $\beta$-catenin as the cells are migrating to deeper cushion areas. These features are more difficult to appreciate in double or single channel images (Fig. 1A, B).

### *Localization of Snail with a mouse monoclonal antibody on mouse tissue*

We have used a mouse monoclonal antibody to locate nuclear Snail in mouse embryo tissue (Fig. 1E–H). As it is well known, the use of secondary antimouse IgG antibodies causes a strong background in mouse tissue, sometimes fading the relevant signal. In fact, most of the staining shown in Fig. 1F is nonspecific background. The image obtained with our method (Fig. 1H) shows nuclear localization of Snail in the perineural plexus cells (arrows), together with complete elimination of the unspecific background. Nuclei of cells nonexpressing Snail virtually disappear from the processed image (arrowheads).

### *Colocalization of HNF1/PCNA*

Developing liver in midgestation mouse embryos is constituted of hematopoietic cells, hepatoblasts, endothelial, mesenchymal and other types of cells. For studies of cell proliferation in the developing liver it may be important to distinguish between these different cell populations. We have applied our technique to specifically identify proliferating hepatoblasts. With this purpose we performed colocalization of the hepatoblast-specific transcription factor HNF1 (Fig. 1J) with the proliferation marker PCNA (proliferating cell nuclear antigen, Fig. 1K). As shown in the figure, proliferating hepatoblasts are forming clusters (arrows in Fig. 1L) and can be distinguished from all the remaining proliferating cells. Other hepatoblasts show lower levels of PCNA antigen, suggesting a slower proliferation rate (arrowheads). These 'hot spots' of proliferating hepatoblasts cannot be recognized in the original confocal image (Fig. 1I).

## Conclusion

Accurate antigen colocalization on tissue sections became possible with confocal microscopy and the use of different fluorochromes. However, at present, a standard of colocalization has not been hitherto proposed. We have shown, through three examples, a simple method to stress nuclear localization of proteins and antigen colocalization. We think that our method, due to its simplicity and the use of a public domain, worldwide utilized software, could be widely accepted and used for colocalization purposes. On the other hand, the grey scale images obtained with this technique can easily be used for quantification of the results obtained, rendering quantitative analysis more reliable.

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