Digital Image Subtraction, Blue Filter, Enhancement (DISBE): A new approach for quantitative immunohistochemical analysis in light microscopy

V. Bernardo¹, M. Farina², L. Silva³ and U. Lins⁴

¹ Divisão de Genética, CPQ, Instituto Nacional de Câncer
Rua André Cavalcanti, 37 – Centro, 20231-050 - Rio de Janeiro, RJ – Brazil
² Laboratório de Biominalização, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro
CCS, Bloco F, Sala F2-027, Av Carlos Chagas Filho, 373 - Cidade Universitária, 21941-590 - Rio de Janeiro, RJ – Brazil
³ Departamento de Estatística, Instituto de Matemática, Universidade Federal Fluminense
Rua Mário Santos Braga s/n – 7º andar Campus do Valonguinho, Centro 24020-140 - Niterói, RJ – Brazil
⁴ Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro
CCS, Bloco I, Av Carlos Chagas Filho, 373 - Cidade Universitária, 21941-902 - Rio de Janeiro, RJ – Brazil

We recently introduced a new image processing procedure - Digital Image Subtraction, Blue Filter, Enhancement (DISBE) - enhancing visual perception of immunostaining. This procedure has a performance rating similar to both human assessment and bright field imaging in quantitative immunohistochemistry. Our approach is based on image subtraction, using blue filtered images of DAB/hematoxylin-stained slides. Here, we present a detailed description of this technique including a tutorial and original data on pixel sampling, camera dark noise and performance of dichroic filters.

Keywords immunohistochemistry; computer-assisted image analysis; microscopy; computer-assisted image processing

1. Introduction

Immunohistochemistry is a worldwide used technique, both in diagnostic and research laboratories. The analysis of immunostains can be qualitative or quantitative. In qualitative analysis, the most widely used method, the researcher is interested in identifying the presence or absence of the target antigen (e.g. diagnostic routine). In quantitative analysis a more sophisticated scoring procedure than qualitative assessment is needed because knowledge of the degree of positivity and the fraction of positive cells is required. The quantitative information is valuable to analyze certain antigens, especially those predictive of response to treatment or related to prognosis (1).

The human visual system is mainly a comparative rather than a measurement system. Computer-based measurements should be able to outperform humans in measurement tasks, especially if controlled imaging conditions are established (2). In fact, digital image analysis (DIA) has shown superior performance over human-based quantitative analysis and it enables detailed analyses to be done rapidly and efficiently (3,4).

We introduced recently a new computer-assisted image processing procedure, called DISBE, for Digital Image Subtraction, Blue filter, Enhancement (5). The DISBE approach is based on image subtraction. Dark noise-corrected blue filtered images of background and specimen are subtracted. This yields a dark background because non-stained areas and background have similar RGB levels. In addition, illumination inconsistencies are removed (6). This yields a new visual perception of immunostains, where strongly immunostained areas are represented by a bright blue color, descending to dark blue levels as immunostaining intensity turns dusky. Counterstaining is represented by green levels. In addition, contrast and brightness enhancement completely remove the red channel (that had already been reduced by the blue low pass filter). The number of possible colors in 24-bit color images is reduced from 16,777,216 to 65,536 maintaining our structures of interest. This approach creates an image that aids both human visual observation and digital image analysis (DIA) systems in assessing immunostained slides, delivers a quantitative performance similar to that of bright field imaging, gives discrimination planes with smaller ranges, and allows the segmentation of strongly immunostained areas in DAB/hematoxylin-stained slides, which have a higher probability to be specific immunostaining. Here, we present a detailed description of this technique, from initial image analysis adjustments to specific image-processing details. In addition, original data on analysis of camera dark noise and performance of dichroic filters are presented.

2. Digital image analysis system performance

The optical system should be optimized to achieve ideal image resolution and reproducible performance. The method to assess reproducibility of labeling (or area) indexes produced by DIA systems, the method to create discrimination planes and the procedures for the adjustment of counting tools are described elsewhere (5). Important steps are also described in section 7.1 - Initial procedures.
2.1 Digital image analysis systems

The image analysis system used in DISBE development consisted of a light microscope (Zeiss Axioplan 2 – Zeiss, Jena, Germany) and a CCD color camera (color view XS - SIS, Münster, Germany - 1280 x 1024 pixels) connected to a desktop computer. A color, brightness and contrast test chart was used to calibrate the monitor. The light microscope power knob was adjusted to keep illumination constant at a fixed value (3200K). Camera pixel size was 6.7 x 6.7 μm. The dimensions of the digital microscopic field were 430.8 X 344.7 μm (width X height). The exposure time and gamma were set to 100 μs and 1, respectively. Camera color settings were: R (gain 2.04), G (gain 1.31) and B (gain 1.71). The offset was the same in all channels (-32). This system was called system X.

Other two image analysis systems were used to evaluate camera dark noise. They were called systems Y and Z. The image analysis system Y consisted of a light microscope (Olympus BX51 – Olympus, Tokio, Japan) and a CCD color camera (DP72 - Olympus, Tokio, Japan - 4140 x 3096 pixels) also connected to a desktop computer. Camera pixel size was 6.45 x 6.45 μm. The exposure time and gamma were set to 143 μs and 1, respectively. The image analysis system Z consisted of a light microscope (Olympus BX51 – Olympus, Tokyo, Japan) and a CCD color camera (Q-Color 5 – Olympus/QImaging, Surrey, Canada - 2560 x 1920 pixels) connected to a desktop computer. Camera pixel size was 3.4 x 3.4 μm. The exposure time and gamma were set to 3.7 ms and 1, respectively. Exposure times were set as those that produced the best images of DAB/hematoxylin-stained slides in each system. These two light microscopes had their power knobs adjusted to keep illumination constant at a fixed value (knob value at 9).

Bright field transmission light microscopy was used throughout this work. Images of immunostained slides were acquired by using a 20x/0.5 Plan Neofluar objective lens, after being set up for Köhler illumination on the computer screen. All images were stored as uncompressed files. All light was directed to the camera chip (no prism was used to send light to the eyepiece). Condensers were adjusted to bright field transmission light microscopy and matched to objective lens numerical aperture (NA). The power supply of each system was stabilized.

The image analysis software Image-Pro Plus 4.5 (Media Cybernetics, Silver Spring, MD) was used in all morphometric measurements.

3. Statistical methods

For the statistical analysis, a commercially available software (SPSS 10.0; SPSS Inc., Chicago, IL) was used. The dark noise of cameras was expressed as mean ± standard deviation (mean ± sd) and compared by Repeated Measures Analysis of Variance. Comparisons between cameras were complemented by Tukey test. The performance of dichroic filters was assessed by one-way ANOVA, followed by Tukey test. A value of $p < 0.05$ was considered significant.

4. Dark noise analysis

We evaluated dark noise over time acquiring three sets of dark noise images from each system. These dark noise images were acquired by blocking the illumination cone. Each set was composed of ten images, each one acquired at 20 minutes intervals for 3h. During intermissions, all cameras remained in live mode. Full image histograms were obtained from each dark noise image and the mean RGB levels were compared to assess if these dark noise series were equal or not.

This experiment showed that dark noise was completely different between these cameras (p<0.0001, Table1).

<table>
<thead>
<tr>
<th>System</th>
<th>R</th>
<th>G</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>7.42 ± 0.253</td>
<td>4.51 ± 0.201</td>
<td>5.48 ± 0.154</td>
</tr>
<tr>
<td>Y</td>
<td>0.005 ± 0.001</td>
<td>0.027 ± 0.004</td>
<td>0.096 ± 0.010</td>
</tr>
<tr>
<td>Z</td>
<td>118.32 ± 0.187</td>
<td>87.07 ± 0.127</td>
<td>121.00 ± 0.166</td>
</tr>
</tbody>
</table>

Results from each channel are expressed in gray levels and were kept with decimal places to better depict our findings (mean ± sd).

However, the repeated measures analysis of variance showed that it did not vary over time in each system, neither comparing dark noise images acquired in the same session nor comparing dark noise images from different days (p>0.05). So, it is necessary to acquire only one dark noise image in order to correct all images acquired in the same quantification session. It reduces the workflow and optimizes intermediate image correction procedures.

An almost negligible dark noise was found in system Y and dark noise subtraction is not necessary in this system. If we realize that RGB levels are natural numbers we will note that this image processing step almost does not alter images from system Y, being time consuming. In system Z a strong dark noise was present. The subtraction of dark noise from each image is advisable both in system X and system Z because it is an intrinsic characteristic of these CCD cameras that influence image acquisition (artificially increasing RGB levels). This might influence quantitative results, especially if different research groups (using different DIA systems) evaluate the same antigen by...
immunohistochemistry. Even when we reduced the exposure time to the lowest possible in system Z (1.6ms), the dark noise image remained the same (0’ – R 118.2; G 86.99; B 120.93).

The dark noise image can not be considered as representing exactly the amount of dark noise present in each image of immunostained fields. However, it is a good estimation. Dark noise and shading-corrected images from system X produced indexes with a strong correlation with those from human assessment (5). In addition, dark noise correction produced images with superior quality under human visual observation.

5. Dichroic filters

Here, images acquired without insertion of filters in optical train are called “bright field” images (BRF). Those acquired with insertion of filters were called “blue filtered” images (BF), “violet filtered” images (VF) and “green filtered” images (GF), when a KP 560 blue low pass (Zeiss, Jena, Germany), a KP 425 violet low pass (Zeiss, Jena, Germany) and an IF 550 interference green filter (Olympus, Tokyo, Japan) were used, respectively.

Images from 120 immunostained fields (60 cytoplasmic immunostained areas from only one slide and 60 nuclear immunostained areas from another slide – oral squamous cell carcinoma specimen) were acquired using these four “image types” in system X and stored in its desktop. So, every immunostained field had a bright field image, a blue filtered image, a violet filtered image and a green filtered image. All images were dark noise-corrected. The united discrimination plane method was used to segment immunostained areas in bright field images (5). These areas of interest (AOIs) were stored and used to set discrimination planes in the other three image types. Because the same AOIs were analyzed in all image types, we were able to assess the effect of dichroic filters on RGB levels, producing discrimination planes adjusted for each image type (Table 2). This table shows, as expected, a predominance of one channel on others depending on the type of filter used.

Table 2 Discrimination planes showing the effect of filters on segmentation of DAB-immunostained slides

<table>
<thead>
<tr>
<th></th>
<th>BRF</th>
<th>BF</th>
<th>GF</th>
<th>VF</th>
</tr>
</thead>
<tbody>
<tr>
<td>A R</td>
<td>69-206</td>
<td>8-28</td>
<td>4-20</td>
<td>28-64</td>
</tr>
<tr>
<td>G</td>
<td>31-171</td>
<td>14-97</td>
<td>14-37</td>
<td>4-17</td>
</tr>
<tr>
<td>B</td>
<td>31-125</td>
<td>21-100</td>
<td>5-30</td>
<td>4-12</td>
</tr>
<tr>
<td>B R</td>
<td>75-230</td>
<td>16-30</td>
<td>10-20</td>
<td>26-42</td>
</tr>
<tr>
<td>G</td>
<td>32-224</td>
<td>17-134</td>
<td>14-37</td>
<td>4-21</td>
</tr>
<tr>
<td>B</td>
<td>38-125</td>
<td>23-96</td>
<td>9-39</td>
<td>5-17</td>
</tr>
</tbody>
</table>

Legend: A – Cytoplasmic immunostaining, B – Nuclear immunostaining

The influence of dichroic filters on segmentation of DAB-immunostained areas was evaluated using bright field images as reference. To avoid the border effect we quantified only a central area (87.5% of field area) using a predefined rectangular AOI.

Results showed that only blue filtered images had a performance rating similar to bright field imaging, both in nuclear and cytoplasmic immunostaining. Table 3 shows that only confidence intervals (95%CI) of bright field and blue filtered images superimpose, reflecting the similarity between the immunostained area (cells) counted.

Table 3 Quantification of immunostained fields using dichroic filters

<table>
<thead>
<tr>
<th>Immunostained area (60 fields)</th>
<th>BRF</th>
<th>BF</th>
<th>GF</th>
<th>VF</th>
</tr>
</thead>
<tbody>
<tr>
<td>(67373.76 – 72358.91)</td>
<td>69866.33</td>
<td>72902.54</td>
<td>1586.92</td>
<td>28984.84</td>
</tr>
<tr>
<td>Immuno stained cells (60 fields)</td>
<td>144.27</td>
<td>149.33</td>
<td>22</td>
<td>2.45</td>
</tr>
</tbody>
</table>

Results are expressed as mean (95%CI). Immunostained area is expressed in µm². The mean number of counted cells was kept with decimal places to better depict our findings.

Table 4-A shows that the use of filters was responsible for the variation observed both in cytoplasmic and nuclear immunostaining quantification. The Tukey test revealed that only blue filtered images did not produce labeling (area) indexes different from bright field images (Table 4-B). These results suggest the exclusion of both interferential green filter and low pass violet filter. They should not be used to quantify immunostains using DAB/Harris’ hematoxylin-stained slides.
Table 4 Analysis of variance assessing the effect of dichroic filters on immunostaining quantification

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>IMMUNOSTAINED AREA</th>
<th>IMMUNOSTAINED CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sum of Squares</td>
<td>df</td>
<td>Mean Square</td>
</tr>
<tr>
<td>Filters</td>
<td>2.12 $E+10$</td>
<td>3</td>
</tr>
<tr>
<td>Error</td>
<td>2.02 $E+09$</td>
<td>20</td>
</tr>
<tr>
<td>Total</td>
<td>2.32 $E+10$</td>
<td>23</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>BRF</th>
<th>GF $\Delta = 69.279.4$</th>
<th>BF $\Delta = -3.036.2$</th>
<th>VF $\Delta = 40.881.5$</th>
<th>$\Delta = 122.3$</th>
<th>BF $\Delta = -5.1$</th>
<th>VF $\Delta = 141.8$</th>
<th>$\Delta = 19.5$</th>
<th>$\Delta = 146.9$</th>
</tr>
</thead>
<tbody>
<tr>
<td>p &lt; 0.0001</td>
<td>p &gt; 0.05</td>
<td>p &lt; 0.0001</td>
<td>p &gt; 0.05</td>
<td>p &lt; 0.0001</td>
<td>p &gt; 0.05</td>
<td>p &lt; 0.0001</td>
<td>p &gt; 0.05</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>GF</td>
<td>$\Delta = -71.315.6$</td>
<td>$\Delta = 27.397.9$</td>
<td>$\Delta = 0.0007$</td>
<td>$\Delta = -127.3$</td>
<td>$\Delta = 0.0007$</td>
<td>$\Delta = 0.0007$</td>
<td>$\Delta = 0.0007$</td>
<td>$\Delta = 0.0007$</td>
</tr>
<tr>
<td>p &lt; 0.0001</td>
<td>p &gt; 0.05</td>
<td>p &lt; 0.0001</td>
<td>p &gt; 0.05</td>
<td>p &lt; 0.0001</td>
<td>p &gt; 0.05</td>
<td>p &lt; 0.0001</td>
<td>p &gt; 0.05</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>BF</td>
<td>$\Delta = 43.917.7$</td>
<td>$\Delta = 0.0001$</td>
<td>$\Delta = 0.0001$</td>
<td>$\Delta = 0.0001$</td>
<td>$\Delta = 0.0001$</td>
<td>$\Delta = 0.0001$</td>
<td>$\Delta = 0.0001$</td>
<td>$\Delta = 0.0001$</td>
</tr>
<tr>
<td>p &lt; 0.0001</td>
<td>p &gt; 0.05</td>
<td>p &lt; 0.0001</td>
<td>p &gt; 0.05</td>
<td>p &lt; 0.0001</td>
<td>p &gt; 0.05</td>
<td>p &lt; 0.0001</td>
<td>p &gt; 0.05</td>
<td>p &lt; 0.0001</td>
</tr>
</tbody>
</table>

Legend: BRF – Bright field images; BF – Blue filtered images; GF – Green filtered images; VF – Violet filtered images

We hypothesize two reasons for this phenomenon:

1. The green interference filter allows only a short range of wavelengths to reach the specimen. DAB immunostained areas and hematoxylin stained areas are then represented by similar RGB levels. Specificity correction also excludes DAB-stained areas in order to exclude hematoxylin-stained areas, decreasing significantly the quantified area. The violet filter also allows a short range of wavelengths in visible spectrum, but other wavelengths can reach the CCD chip. Several isolated pixels were segmented in violet filtered images, increasing the quantified area. When cells were counted, these isolated pixels did not reach the minimum size needed to be automatically counted as “cells” [20.5 $\mu m^2$ (5)], producing very low countings. In nuclear immunostaining quantification, the green filtered images also had a poor performance. So, filters with short wavelength ranges should not be used in quantitative analysis by DIA.

2. The blue filter matches the color of hematoxylin counterstaining. This assumption is based on evidences that when a filter increases those levels that match counterstaining it increases contrast of areas that have a different RGB distribution. If this second hypothesis is true we have a new paradigm and the subtractive approach could be used in other combinations of chromogens, using different counterstainings and filters (e.g. fast red + Harris’ hematoxylin or DAB + methyl green).

6. Pixel sampling (Castleman criterion)

Castleman (1987) established a criterion to analyze the image acquisition procedure, enabling a computed-assisted analysis of reliable specimen images. The Castleman criterion addresses the question of how many sample points (pixels) per scan line are required to digitize an image from a light microscope, in order to maintain the accuracy of the quantitative data extracted (7). To apply the Castleman criterion in system X (1280 pixels per scan line), using the four image types already described, a green wavelength ($\lambda = 0.55 \mu m$) was used as the mean wavelength seen by the human eye in bright field images [visible range: 400 - 700 nm; (8)]. For the blue, violet and green filters we considered the mean wavelength ($\lambda = 0.48 \mu m$ - range: 400 - 560 nm; $\lambda = 0.4125 \mu m$ - range: 400 - 425 nm and $\lambda = 0.55 \mu m$ - range: 540 - 560 nm, respectively). All but violet filtered images were adequately digitized by image analysis system X, according to the Castleman criterion (Table 5).
Our results showed that violet filtered images had the worst performance. This image type was outside Castleman criterion, showing statistically different indexes from bright field imaging (p<0.0001), low countings and a poor segmentation pattern. Nowadays, modern cameras have more pixels per scan line, so probably this issue will not be a limiting step in a near future. Green filtered images had also a poor segmentation pattern and in nuclear immunostaining produced a labeling index similar to violet filtered images (Table 4B). Probably, a poor segmentation pattern is generated using short wavelength ranges (observed in both violet and green filtered images). This yielded counterstaining and immunostained areas with similar RGB values. Subsequently, specificity correction excluded both positive and background areas.

7. DISBE tutorial

In the next section we describe in details this technique and show the effect of this procedure on images from different image analysis systems.

7.1 Initial procedures

The light microscope should be properly aligned and all components should function properly to achieve maximum efficiency. A Bertrand lens or a telescope eyepiece should be used to evaluate conjugate aperture planes and visualize the lamp filament after removal of the diffuser screen. The reflection image of the filament is adjusted until the vertical lines of the primary and reflection images of the filament loops are interdigitated (9).

The illumination pattern after the alignment procedure can be evaluated with a neutral density filter (e.g. S&K coated filter ND2, Shiro Photo Corp, Tokyo, Japan). This filter was coverslipped using an Eppendorf CELLocate coverslip (Eppendorf North America Inc, New York, NY) to obtain a Z-dimension reference. A surface plot of the neutral density filter image can be used to evaluate illumination patterns, depicting the illumination pattern visually (Fig 1).

Our slides were 3-μm sections from paraffin blocks that had peroxidase labeling developed with DAB for 3 minutes. This was followed by counterstaining with Harris’ hematoxylin for 40 seconds.

Fig. 1 Surface plot of illumination pattern (system X). a) As expected after alignment, the surface plot of neutral density filter image shows the typical aspect, with image borders less illuminated than the central area. b) a line profile of the same neutral density filter image showing RGB values along image width {X axis shows image width [0 - 430.8 μm]; Y axis shows intensity [0 – 255]; colors depict RGB channels}.
1. Apply the Castleman criterion to assess the digitalization procedure in your system. Quantitative immunohistochemical analysis should be made with semiapochromat or planapo 20x objective lenses because these lenses are better corrected. In addition, 20x lenses allow a larger field of view than 40x lenses, maintaining morphological details and reducing the workflow;

2. Microscope adjustments: (1) match the NA of the condenser to the 20x objective NA; (2) always use Köhller illumination, using the computer screen as reference; (3) set microscope power knob at a fixed position (this value should be the one that produces the best images of DAB/hematoxylin-stained slides in the system - see step #3);

3. Define camera color settings, exposure time and other camera adjustments that produce the best images of DAB/hematoxylin-stained slides in the system, using bright field images as reference. Always keep gamma at 1, to ensure linearity between the input and output signals from the CCD camera;

4. Make sure that these settings are always reproduced before every image acquisition session (when these images are expected to be used in a quantitative analysis);

5. Assess the reproducibility of indexes produced by your DIA system using the method described in our original publication. Briefly, this is done comparing labeling (area) indexes from six randomly selected fields imaged by the digital system over time, using repeated measures analysis of variance to assess the reproducibility of the indexes produced (5).

7.2 DISBE steps

7.2.1 Image acquisition

1-Clear and put the slide on the microscope stage;
2-Set microscope power knob to the predetermined position (the one that produced the best images of DAB/hematoxylin-stained slides);
3-Check camera settings;
4-In low power (4x or 10x) locate areas of specific immunostaining;
5-Swing in the 20x lens and refocus on the tissue;
6-Match the NA of the condenser to the 20x objective NA;
7-Set Köhller illumination on the computer screen;
8-Block the illumination cone;
9-Acquire the dark noise image, which is going to be used to correct all images further acquired in the same quantification session. Steps # 8, #9 and #10 must be done only once per image acquisition session;
10-Unblock the illumination cone;
11-Insert the KP 560 blue low pass in the optical train, placing it on collector lens;
12-Acquire the background image (moving the stage to a coverslipped area without specimen);
13-Acquire images of immunostained fields. Immunostained fields should be selected randomly inside areas of specific staining. A random number table can be used to ensure a random stage movement;
14-Remove the slide from the stage;

7.2.2 Image acquisition

15-Open the Image-Pro Plus image analysis software;
16-Open the dark noise image and all images of the immunostained slide (background and immunostained fields; these are all blue filtered images);
17-Subtract dark noise image from background image and immunostained fields images, using the tool "operations" from "process" menu (these dark noise-corrected images will be used in the next DISBE steps);
18-Subtract dark noise-corrected images of immunostained fields from the dark noise-corrected background blue filtered image;
19-Increase contrast and brightness digitally up to span the entire range in G and B channels of the RGB color space, using the tool "contrast enhancement" from "enhance" menu. When an image is opened with Image-Pro Plus, its intrinsic brightness and contrast receive a value of 50. In system X, contrast and brightness were digitally increased to 88 and 78 (respectively) in nuclear immunostaining and to 88 and 65 in cytoplasmic immunostaining;
20-Apply these changes and save the DISBE images for subsequent quantitative procedures.
Figure 2 shows the same immunostained field as imaged by the DISBE procedure using the different image analysis systems described here. Using adjustments of contrast and brightness designed for each DIA system, they produced similar DISBE images of this field. Note that these different DIA systems produced different field sizes under the same magnification (20x objective lenses).

1) **System X**

![System X Bright Field](bright_field_x.png) ![DISBE X 78 brightness, 88 contrast](disbe_x_78_contrast.png)

**System Y**

![System Y Bright Field](bright_field_y.png) ![DISBE Y 78 brightness, 88 contrast](disbe_y_78_contrast.png) ![DISBE Y 88 brightness, 87 contrast](disbe_y_88_contrast.png)

**System Z**

![System Z Bright Field](bright_field_z.png) ![DISBE Z 78 brightness, 88 contrast](disbe_z_78_contrast.png) ![DISBE Z 88 brightness, 93 contrast](disbe_z_88_contrast.png)

**Fig. 2** DISBE approach on different DIA systems

### 8. Conclusion

Here, we showed experiments that were part of the development of the DISBE image processing approach and discussed variables that should be considered during image analysis experiments. We also demonstrated that the DISBE approach can be used in different image analysis systems, even when a strong dark noise is present. We are supposed to encourage other research groups to extract reliable quantitative information from the valuable and widespread used immunohistochemical technique.

**Acknowledgements** The support by CNPq and FAPERJ is gratefully acknowledged.
References


