EUROMEDITERRANEAN BIOMEDICAL JOURNAL

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Review

MULTISPECTRAL MICROSCOPY AND COMPUTERIZED IMAGE ANALYSIS: A METHOD TO OBTAIN MORE RELIABLE AND REPRODUCIBLE IMMUNOHISTOCHEMISTRY RESULTS

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Summary

The use of image analysis methods has allowed us to obtain more reliable and reproducible immunohistochemistry (IHC) results. Wider use of such approaches and simplification of software allowing a colorimetric study has meant that these methods are available to everyone, and made it possible to standardize the technique by a reliable systems score. Moreover, the recent introduction of multispectral image acquisition systems methods has further refined these techniques, minimizing artefacts and easing the evaluation of the data by the observer.

Introduction

Immunohistochemistry is used for identifying specific antigens, as well as for highlighting typical markers of various diseases by pathologists. Since its introduction to scientific research, this technique has suffered from some serious limitations inherent to its execution. Even though the steps that precede the evaluation of the results can be standardized [1,2], it is much harder to reproduce the experiment and evaluate the data obtained. In fact, the interpretation of an IHC result depends on the operator and his/her experience. There are many factors that influence IHC observation, starting from the type of microscope, the colour temperature of the light source and the type of dye used, the visual ability of the operator and last, but not least, the optical nonlinearity of the reactions. Indeed, if it can be stated that an increase in the antigen concentration also increases the intensity of staining at low concentrations, this is not true at higher concentrations of antigen. Therefore, our optical perception does not allow for a proper assessment of the actual reactivity.

In order to overcome the limitations described above, in the past few years there has been a collective effort to standardize the IHC technique as much as possible both in the preparation phase and during the actual reaction and subsequent interpretation of the data[1,2]. Digital imaging systems, accompanied by increasingly sophisticated software for image analysis for the past 15 years so, have offered a significant means of support to achieve this goal. Image analysis is a method that allows the assessment

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Received: *8th December, 2015 — **Revised:** 15th December, 2015 — **Accepted:** 21th December, 2015*

of both the number of positive cells and the intensity of the reaction as colorimetric parameters derived from a digital image through a more or less specific software.

Image analysis

The main issues with the evaluation of immunohistochemical reactions depend, as previously pointed out, from several, mainly subjective, parameters. What one operator considers a positive test can instead be absolutely negative for another, depending from the individual colour perception and visual capacity, the observation conditions, etc.

The fact that IHC reactions do not have a totally linear yield from the chromogenic point of view must also be taken into account. The reactions are effectively linear at low antibody concentrations, but if the substance sought is present in greater concentrations, the chromogenic response appears non-linear; therefore, after a certain threshold (very low for that matter) it is no longer possible to reliably discriminate the amount of substance present within the cell.

On the other hand, immunofluorescence tends to produce an almost linear response, with the higher concentration of the substance corresponding to an actual increase in fluorescence. For this reason, the latter technique is being increasingly used for image analysis.

Since the early nineties, the use of digital images in microscopy has become increasingly popular and this has resulted in an ever-growing number of information being obtained from the samples studied. Clearly, in pioneering systems such as the first methods based on the acquisition of videos and non-photographic images, the low resolution of the frames acquired did not allow accurate colorimetric assessments. Nonetheless, the introduction of some software, such as the Nikon Lucia M, can be considered as a true revolution in the field of IHC analysis, since they helped to simplify both the count of positive cells and the evaluation of the results by introducing the concept of colorimetric quantification. This type of evaluation allowed researchers to obtain an idea of the amount of the substance present within various cytotypes, even if just in an approximate manner. Due to the low resolution it was agreed that binary (2-bit black and white) or grayscale images, that appear to be more detailed at 8-bit or 256 shades of gray, should be used. It is important to note that the human eye can distinguish about 70 shades of gray, so the use of images at 256 shades requires a software that can carry out an accurate evaluation of the intensity of the reaction. The use of the b&w images ruled out the differences linked to the different types of chromogens used and allowed greater standardization of the image analysis techniques [3,4].

To overcome the abovementioned non-linearity of IHC reactions and the unfeasibility of objective evaluation by individual operators (phrases like "diffusely reactive" or a "high" or "low" immunoreactivity are absolutely useless and inaccurate) and to correctly identify the presence and distribution of a substance, it is of paramount importance to use an image analysis system that uses a score to quantify the reactivity of a sample, allowing us to answer these basic questions:

- Is the reaction positive based on the controls?
- If yes, what is the degree of intensity and is it correlated with the quantity of the antigen?
- Where is the reactivity localized?

The development of systems for image acquisition has changed some parameters in use today, so if a few years ago was essential to use binary, or at most 8-bit images, as explained before, today's higher resolution also allows the use of colour images.

Digital colour images are based on some basic parameters, and, in particular, on the histograms related to the combination of basic colours, whether RGB (red, yellow, blue) or CMYK (cyan, magenta, yellow, black). The profiles of these histograms vary appreciably according to the specific colour being examined. In particular, it has been noted[5] that the CMYK profile allows a more progressive evaluation, and that using the yellow channel in particular produces comparable results independently from the observer and is applicable to several types

of chromogens as it is sensitive to minor variations in IHC intensity with a linear scale enabling the obtainment of an accurate and easily reproducible score. Considering that each channel is divided into 256 shades, a specific value can be assigned in a linear progression to a variable score from 1 to 7, where 1 corresponds to the absence of colour or 0, and 7 to the maximum value obtainable according to the chromogen used (Figure 1).

Concurrently with the development of imaging systems, simplifying analysis software has allowed the diffusion of the IHC technique that is now available to anyone with minimal experience in the use of common image editing programs such as Photoshop [6,7], whose latest version also contains an image analysis section, where a few simple commands enable the user to conduct a series of surveys ranging from the count of positive cells to the quantification of colorimetric values to be scored [4,8,9].

Multispectral image analysis

Recently, the IHC approach based on the CMYK profile-derived score has been complemented by a technique involving the acquisition of multispectral images. This method, originally used to improve the quality of images taken from satellites and since applied in different research fields, from geological evaluation of the soil to archaeological studies where multispectral imaging is used for evaluating the presence of settlements in the subsoil, is based on the possibility to overcome, through specific technical expedients, the restrictions of human vision which is limited by light- and observation condition-dependent colour perception. In physics, a colour stimulus is unequivocally defined by the "intensity" it assumes in the spectrum of visible light. Therefore, each colour is associated with a "spectral curve" built according to the energy radiated by the individual wavelengths [10]. To highlight these curves, it is necessary to acquire images that take into account what is defined as "reflectance" in physics terminology, or the ratio of incident radiation which a surface is able to reflect. A multispectral imaging system thus allows us to determine the reflectance in the visible spectrum, and faithfully reconstruct the colours in the image. The multispectral systems use special optical filters, sensitive to different wavelengths in the visible spectrum, and subsequent software systems able to reconstruct the reflectance curves at each point of the scene, to produce an image whose various colorimetric components can be studied in detail.

In practice, the multispectral analysis systems comprise of a set of filters that allow the distinction of a single colour through using one such filter set for a certain wavelength in the visible range (Figure 2). A "cube" of spectral data is obtained by selecting one wavelength after the other, acquiring an image for each and successively reconstructing it by means of a special software. The possibility to capture images at different wavelengths means that one or more of them can also be subtracted from the final image, thus highlighting wavelengths specific to a given chromogen or fluorochrome. The potential applications

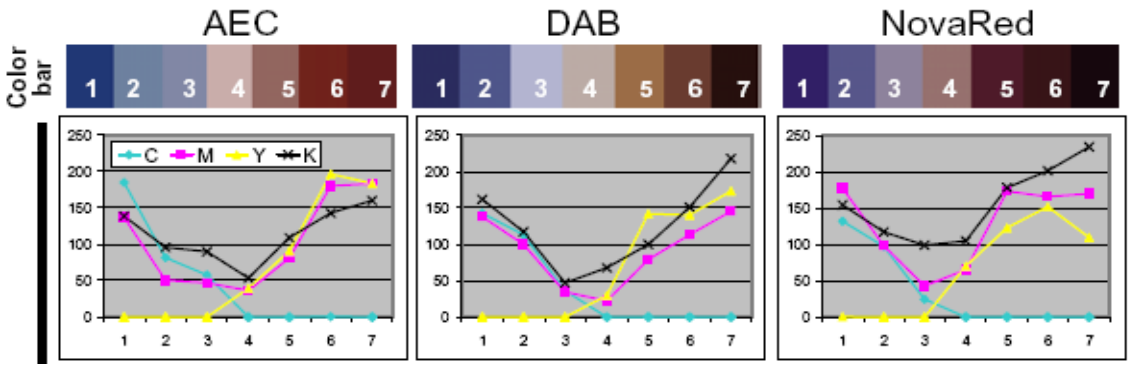

Figure 1. CMYK profiles for the most common chromogens

for this technique are therefore varied. In particular, in the case of fluorescence images, the ability to separate the various wavelengths allows the removal of all or most of the autofluorescence of the tissues, with obvious benefits for the evaluation of results related to a specific reactivity. In traditional immunohistochemistry the use of this method allows to isolate and highlight, by means of a process called pseudofluorescence (producing negative and well contrasted images), the presence of a specific chromogen, its localization and subsequently its intensity (Figure 3-4-5). Moreover, it is also possible to separate and isolate different chromogens used to evaluate a co-localization of different antigens within

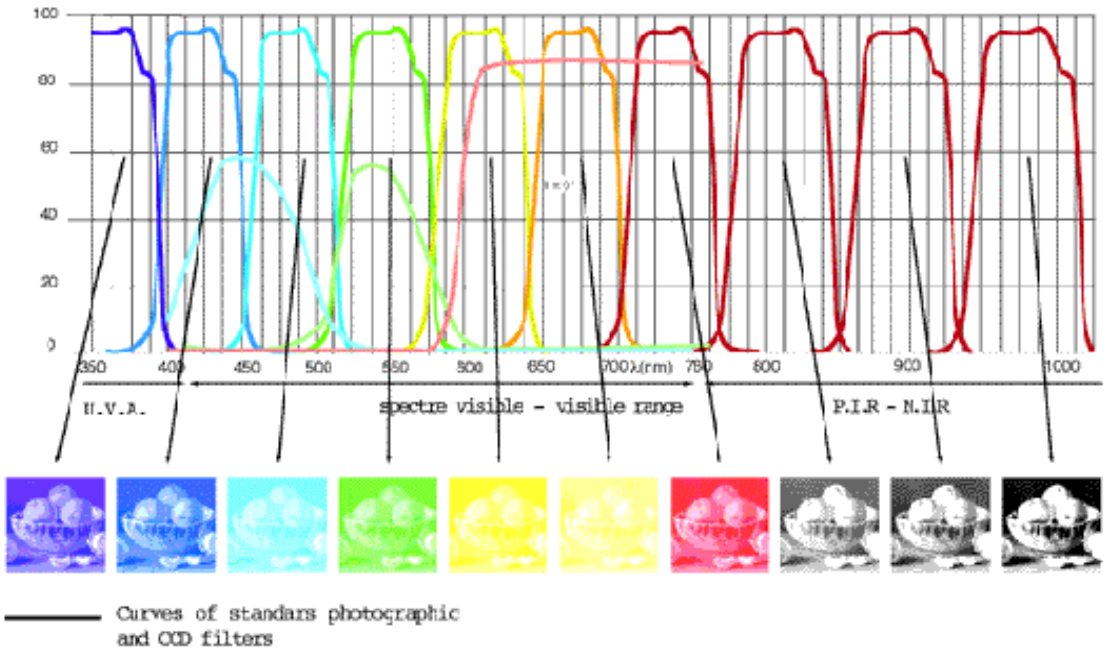

Figure 2. Spectral curves and filters

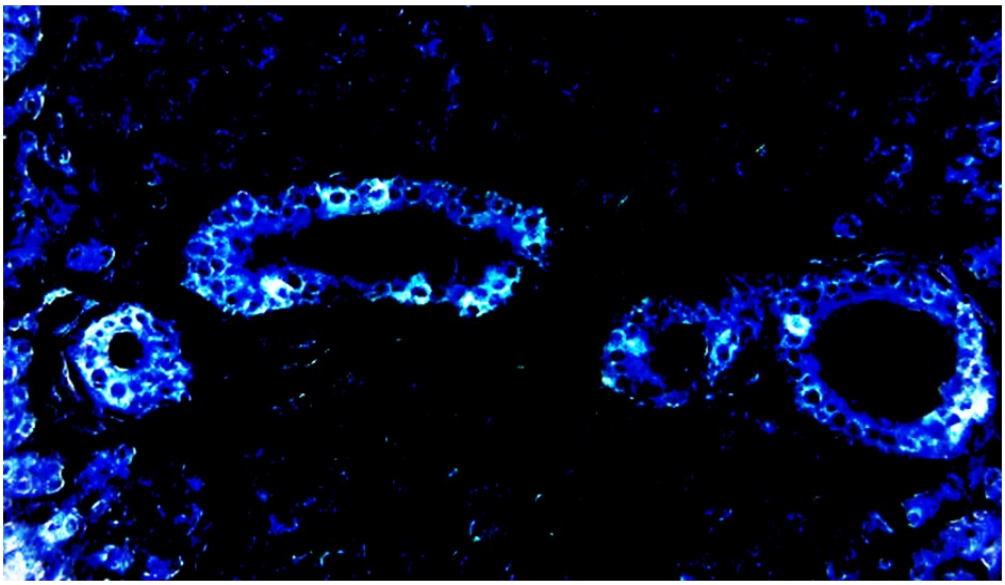

Figure 3. Multispectral acquisition - leptinergic positivity (AEC chromogen) in ductal epitheliocytes – multispectral image – 10X

a single cell or tissue[11,12,13]. Following the acquisition phase, the application of a selective separation of the various spectra by phasing out the ones that do not match the chromogens under evaluation allows the transformation of the image according to the CMYK profile, and thus the evaluation of the yellow channel to obtain the previously described linear colorimetric curve to score for the evaluation of reaction intensity, indirectly quantifying the presence of the antigen.

Conclusions

The IHC approach is still useful to visualize the presence of specific antigens in cells. Combining this method with image analysis and multispectral analysis enables us to obtain reproducible results and minimize the objective limitations determined by different observers. The possibility to use the score-based quantification technique[14,15,16,17,18,19,20] to determine the amount of antigen present within the field of the microscope, together with molecular biology, allows us to have a more complete and certainly more realistic view than using either of these methods separately.

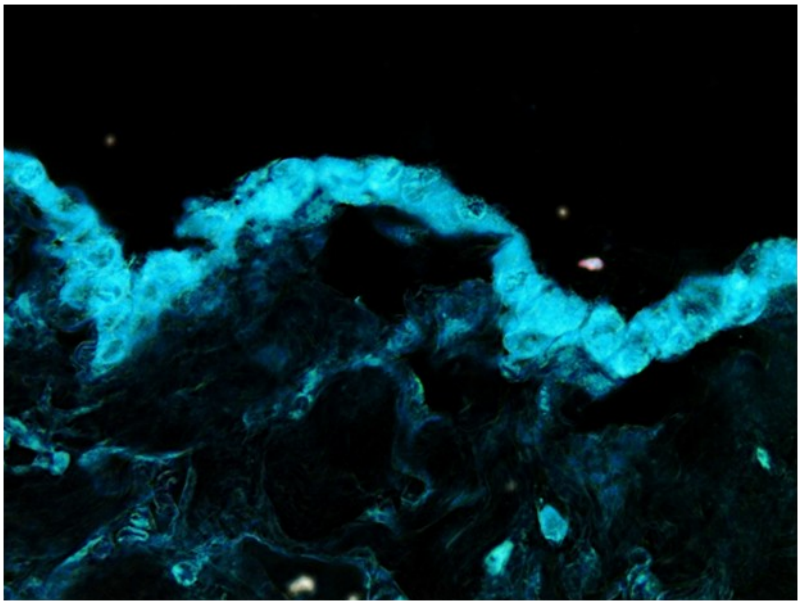

Figure 4. Multispectral acquisition - MMP9 positivity (AEC chromogen) in endothelio-cytes from human dilated aortic aneurysm – multispectral image – 40X

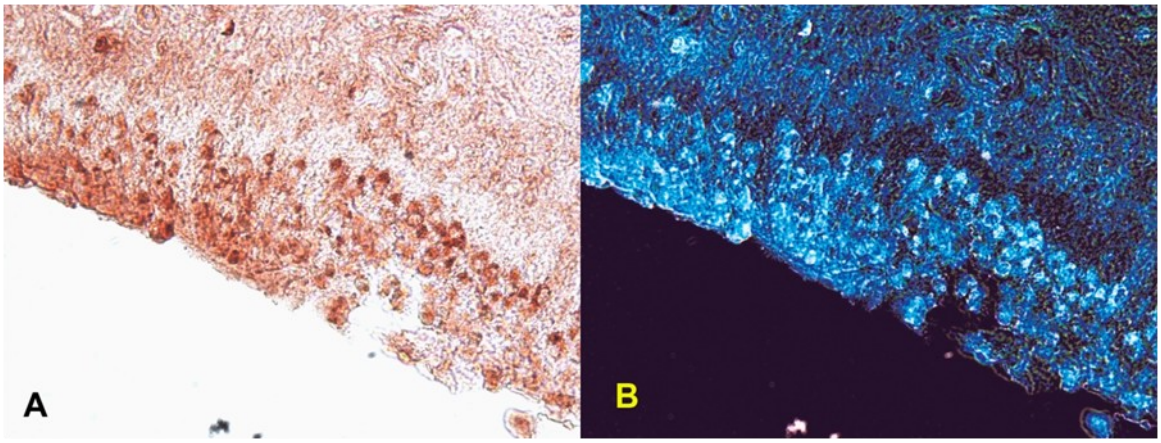

Figure 5. Comparison between traditional microscopy (A) multispectral imaging (B) - MMP9 positivity (AEC chromogen) in dental pulp cells – 40X

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