Microscopic image analysis using computer-assisted methodology to quantify immunostained receptors

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Image analysis is a rapidly expanding field, with large diversity of applications in life sciences, including basic and clinical research, biology, etc. Since most of images in life science are acquired through microscopy, procedures and methods for image analysis present some specificities. In this chapter, an overview on the fundamentals of imaging, processing, staining and analysis in biological tissues is presented guiding the reader through the basic and advanced knowledge of major quantitative image analysis approaches. A validated semi-automated computer assisted image analysis to quantify immunostained receptors in vascular tissue sections (specimens that might not fill the entire image filed of the microscope) is described. This methodology provides an objective description of the differences in receptors staining patterns among sets of tissues and comparison among experimental groups. With that, quantitative evaluation of immunostained receptors in tissue sections provides information about receptor imbalance and/or overexpression that might contribute to a more accurate elucidation of their putative physiological and/or pathophysiological role. In summary, microscope image analysis using computer-assisted methodology to quantify immunostained receptors would be pertinent to applications in both basic and clinical studies.

Keywords: immunostained receptors; computer-assisted image analysis

1. Introduction

Immunohistochemistry is one of the techniques currently used in many laboratories, both for diagnostic and research using increasingly specific antibodies and labelling techniques to identify and map the distribution of a specific antigen. It is easy to standardize but the interpretation relies solely on subjective visual estimation and yields just qualitative results. Qualitative analysis is a task for which human eye is well suited: qualitative data is based on pattern recognition (overall arrangements of elements within the specimen), requiring highly trained individuals. Nevertheless, qualitative analysis may present limitations, for instance, when it is required the precise description of differences in staining patterns among sets of tissues and/or cells, comparison of staining among experimental groups, etc.

To circumvent these limitations, some form of quantification must be performed. The human eye, in spite of being a remarkable highly adaptable sensor, is not well suited for quantitative functions (such as estimation of linear measurements, areas or density of stain, etc). Quantitative analysis methodologies are traditionally based on the Cavalieri's principle [1,2]. It is possible, therefore, to estimate some specimen's parameters (such as area of a total image occupied by a particular component). However, this type of quantitative analysis presents some limitations that should be carefully considered before initiating experiments: i) researcher subjectivity - results depend on the individual interpretation of counting or not, intersections between imposed grids (calibrated grids in which different scale possibilities are available) and the staining; ii) time-consuming, point counting procedure requires high number of specimens for analysis making this a lengthy process; iii) the use of point counting methodology to derive measures of the 3D structure of cell and/or tissue specimens may provide misleading information since the probabilities used in mathematics assume that the entire volume of the specimen is well reflected in the portion measured, however, in general, in cell/tissue 3D structures this is not the case, due to, for instance polarization of cells and tissues/organs arrangement [3].

In the last decades important advances in digital imaging and analysis systems have been described, allowing quantitative measurements to be acquired quickly and efficiently in diverse specimens from a wide variety of disciplines, including life sciences. Acquiring images from cells and/or tissues often requires microscopy and, subsequently, procedures and methods should be carefully chosen to guarantee an adequate image analysis. In the literature there are descriptions of the best suited procedures for counting pixel number [4] or measuring areas [5], often relying on the ability to separate or segment a structure of interest from its background. However, few studies have considered the quantitative characterization of a feature such as receptors. In this chapter, an overview on the fundamentals of imaging, processing, staining and analysis in biological tissues will be provided with particular emphasis on immunostained receptors in sections of vascular tissues.

2. Specimen fixation and processing

The quality of an image dataset mainly depends on two aspects: the correct processing of the sample and the microscope performance. Sample processing, in turn, depends on several factors such as, fixation, loading of

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fluorescent dyes or immunostaining, and the correct mounting of the specimen. The specimen calibration step (performed by the computer), ideally requires homogeneous specimens in chemical, dimension and structural features. However, in life sciences research the majority of specimens are of biological nature with specimens formed by numerous cells (some of them different in nature), arranged in tissues and organs, having lumen (or not), presenting specific thickness, etc. These may confer a heterogeneous composition to biological specimens, which makes, on computer assisted image analysis, the specimen calibration step a difficult procedure. Nevertheless, this is a crucial step since an inadequate calibration can compromise the analysis and data validation. In summary, selection of specimens should ensure, as much as possible, uniformity between them; processing should guarantee identical structure features (such as thickness, dimension, etc) of specimens so that subsequent quantification procedures can be reliable.

In addition to the general aspects described, specimen fixation, processing and incubation buffers are other relevant aspects that should also be considered in quantitative morphological studies. Artefacts due to dimensional changes of the tissues during specimen preparation must be recognized and minimized (e.g. artefacts due to fixation). Fixation is a process that can prevent autolysis and displacement of cell constituents (such as proteins, enzymes, antigens), contributing to stabilize cellular materials by avoiding the deleterious effects of subsequent procedures, and may facilitate classic staining or immunostaining [6]. However, fixation can also potentially reduce the immunostaining signal depending on the fixative chosen and/or duration of the fixation process [7]. In this sense, the use, when possible, of frozen sections instead of wax-embedded sections, can circumvent these issues.

Two main types of fixatives are available: cross-linking and coagulating fixatives. Coagulating fixatives precipitate proteins by breaking hydrogen bonds in the absence of protein cross-linking (e.g. ethanol). However, due to their little stabilizing effect over the protein hydrophobic structure, most of these fixatives lead to inadequate cellular preservation [6]. By opposition, cross-linking fixatives (e.g. formaldehyde) can preserve many peptides, have little effect on carbohydrates [8] and lipids (in the presence of calcium [9]), allowing, therefore, the general preservation of cellular organelles. The mechanism of these fixatives relies on the formation of additional products (such as reactive hydroxyl methyl compounds) between the fixative and uncharged reactive amino groups (NH or NH2), forming cross-links [10]. However, after fixation, and due to the profound changes that occur in macromolecules conformation, it is possible, in some type of experiments, that the recognition of proteins (antigens) by antibodies become difficult or even impossible [11]. Nevertheless, the deleterious effects ascribed to some of these fixatives can be countered using antigen retrieval procedures. These procedures allow countering the modification in the tertiary structure of proteins (antigens) which may turn them undetectable by specific antibodies. Several techniques to retrieve antigens (altered by fixation) became available, namely heat-induced epitope retrieval (HIER); protease-induced epitope retrieval (PIER) among others (e.g. incubation on strong alkaline solutions, microwave). However, these procedures might alter specimen 3D structure and, therefore, in studies were 3D is crucial, it would be desirable to avoid them. In this sense, penetration of antibodies into paraformaldehyde-fixed tissues has been reported to be much superior to that obtained with glutaraldehyde-fixed tissues. There are also reports describing that cross-linked fixatives combined with heat and/or nonpolar solvents (such as those used in wax-embedding) may alter antigen conformation and prevent antibody recognition of a particular epitope (does not occur with the same epitopes/antibodies when frozen sections are used instead) [12].

It is important to recognize that there is no optimal fixative: cross-linked fixatives, such as formaldehyde and glutaraldehyde, are a common choice mainly due to its low cost and reliability in general histology. Glutaraldehyde has been shown to cause cell shrinkage, probably due to hypertonicity of solutions, which can be minimized by adjusting osmolarity. Paraformaldehyde has been suggested to cause some degree of morphological distortion, which can also be minimized using paraformaldehyde solutions at physiological pH and temperature [13,14]. Fixation with paraformaldehyde, unlike with glutaraldehyde, does not give rise to high levels of tissue autofluorescence and, thus, it is more effective for immunofluorescence studies [15].

Other aspects that should be carefully standardized, in order to guarantee reproducibility, are: 2D plan sections as well as 3D structures should be carefully mounted; immunohistochemistry procedures should follow a rigid protocol (incubation times, temperature, chromogen reaction times, light, humidity, and antibody title) and must be performed in several identical batches.

It is also important to account that pH value changes can compromise the binding of monoclonal antibodies to the respective antigens [7]. The buffer used in immunostained procedures is also crucial [16] and, in most studies, the preferred buffer is the phosphate saline buffer (PBS), particularly when monoclonal antibodies are used. However, its use in alkaline phosphate procedures should be avoided since PBS presents high concentration of inorganic phosphates and can, therefore, act as a competitor inhibitor for alkaline phosphate. In this type of applications, PBS should be avoided and substituted by an appropriate buffer such as Tris buffer [17].

Mounting can also be crucial to obtain reliable and reproducible qualitative and quantitative data. Criteria to select the mounting media must consider the preservation of the specimen structure, the need of preventing optical aberrations and retarding photobleaching. For instance, shrinkage of the specimen can occur when using some mounting media containing polyvinyl alcohol, therefore, this type of mounting media should be ruled out in these applications. Moreover, in studies where the specimen 3D structure is crucial, distortion can also be reduced if a space between the slide and the coverslip is used.

In immunofluorescence studies, the observation process leads to fluorophores consumption which is critical when the parameter to be quantified is fluorescence intensity. Fluorescence intensity, as well as bleaching of fluorophores can be influenced by a variety of factors such as mounting fluid, power of excitatory light, etc. Photobleaching is an important issue in fluorescent microscope, particularly in Laser Scanning Confocal Microscopy (LSCM) [18]: the higher power and the focused beam induce enhanced fading of the specimen. This can be minimized by changing some of the experimental conditions related with fluorophores: using antifade agents in mounting media; using fluorophores resistant to bleaching; increasing fluorophores concentration, etc.

3. Imaging immunolabelled specimens

The practice of histology relies on the use of classic staining procedures. There is a wide range of staining chemicals of synthetic or natural origin which leads to better contrast between cellular structures, making possible a better microscope visualization of specimen's features (e.g. hematoxylin, eosin). Other techniques such as imunohistochemistry/immunocytochemistry, *in situ* hybridization (FISH), tissue microarrays or multiplex immunostaining chip can also be use to recognize specific specimen's features. *In situ* hybridization relies on the use of antisense single-stranded DNAs for the particular gene/sequences fluorescent-labelled which can be used as molecular probes. These probes can hybridize with complementary RNA or DNA molecules in the cells and or tissues, being useful to detect a particular gene expression in the cells/tissues, number of copies expressed as well as their localization [18]. In immunohistochemistry/immunocytochemistry, the antigen-antibody reaction cannot be visualized unless it is labelled, i.e., visualization of a particular feature (such as receptors) requires a detection system: labels are attached to the primary, secondary or tertiary antibodies of a detection system [19]. Currently, there are a large number of labels available (enzymes, fluorescent dyes, metals [20,21]) and its selection depends on the type of microscope in use, the characteristics of the ongoing study and on the type of specimen in analysis. With tissue microarray technology it is possible to detect a single antibody (proteins or gene expression) simultaneously in many specimens (on a single microscope slide), while the use of multiplex immunostaining chip allows to simultaneous examine a panel of antibodies in a single specimen [7].

In light microscopy, the most common labels used in iummunohistochemistry/immunocytochemistry are enzymes such as peroxidase or alkaline phosphatase. These enzymes in the presence of a specific substrate and chromogen will produce colored precipitates at the site of antigen-antibody reaction. This can be achieved by using a two-step method: the first layer of antibody is unlabeled; the second layer of antibody is raised against the primary antibody and is labelled [22]. Some studies may benefit from using amplification methodologies which increase sensitivity to antigen detection. There are several examples of these amplifier methods: avidin-biotin (ABC), peroxidase-antiperoxidase (PAP), alkaline phosphatase-antialkaline phosphatase complex (APAAP), tyramine amplification, etc.

Multiple immunostaining can be used in order to elicit, simultaneously, the expression of several antigens present in the same or different cells. This technique became possible with the availability of different detection/amplifier systems such as PAP, ABC, labeled to different chromogens which allow a variety of colored reactions (each color corresponding to a specific antigen).

Apart of the putative occurrence of false positives due to cross-reactivity (which can be solved using several controls and a rigid and well planned protocol) when image and quantitative analysis is the goal, other aspects should be considered, such as, the appropriate selection of chromogens. In fact, quantitative procedures, in multiple immunostaining, require a differential analysis of the color distribution to separate and differentially analyze different chromogens. For instance, using colored filters, selectively excluding transmission of light of a certain wavelength [15], or software tools that allow manipulation of selected areas or features of the object of interest (e.g. Photoshop, Adobe Systems, San Jose, California). Nevertheless, if the researcher has to deal with the presence of two or more type of pixels stained simultaneously, these different pixels must be colored with chromogens emitting on totally separated wavelengths to allow a correct segmentation of these two or more different type of pixels (two or more different objects of interest) in the same section.

In fluorescent microscopy, the most common labels are naturally fluorescent substances (fluorophores, which require UV excitation for optimum results) or dyes, chemicals and antibodies that when added to tissues produce secondary fluorescence of structures (fluorochromes; most of these substances require blue light excitation). The spectral cross-talk between fluorophores is a critical issue that must be solved and may occur due to the very broad bandwidths and asymmetrical spectral profiles exhibited by several fluorophores.

Fluorescence microscopes, including LSCM, are widely used as research tools in life sciences. They enable excitation light to irradiate the specimen and, then, should be able to separate the much weaker re-radiation fluorescent light from the brighter excitation light. Indeed, only the emission light can reach the detector and the resulting fluorescence areas shine against a dark background producing a higher contrast and allowing detection. Additionally, the combination of direct and diffracted light is of critical importance in image formation. The key elements for such manipulation are the back focal plane of the objective (e.g. immersion oil objectives, objectives with high numeric apertures) and the front focal plane of the substage condenser [19,23]. Indeed, microscope resolution, which corresponds to the shortest distance between two points on the specimen distinguished as separate entities, depends on

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the wavelength spectrum of light used (the shorter wavelengths, greater degree of resolving details) and on the optical system.

The exciting radiation is usually in the ultra-violet (UV) wavelength (ca. 360 nm), produced by passing light from a UV-emitting source through the exciter filter. Then, the filtered UV light illuminates the specimen which in turn emits fluorescent light (of longer wavelengths). The light emitted from the specimen can then be filtered through a barrier filter (which does not allow UV light reflected to pass) and re-radiates spherically in all directions, regardless of the direction of the exciting light. In addition to UV, the blue region (ca. 400 nm) is also commonly used in fluorescence microscopy, although longer wavelengths can also be used [19,23]. Above all, the selection of the light source should be appropriated to the type of work to be performed (such as xenon, mercury, halogen lamps).

It is important to highlight the possibility of specimens to contain fluorescing material (primary or auto-fluorescence) in addition to the one in which the observer is interested, contributing with noise. It is, therefore, necessary to filter out all but the specific excitation wavelength in order to discriminate between important and unimportant fluorescence. The use of filters are appropriated to overcome most of these undesirable fluorescent staining. Other sources of noise can still be present such as stray light, statistical noise, etc. Nevertheless, all noise types will contribute to distortion [24]. Nevertheless, in some applications such as plant studies, coal petrography, etc [23], auto-fluorescence can be useful and cannot be considered as noise.

To produce accurate and reproducible evaluations, most microscopes have calibration devices. For instance, eyepiece reticules or grids (calibrated by the use of stage micrometer) can be used to directly measure linear dimensions of objects while areas are generally estimated using a planimeter (mechanical device used to manually trace the outline of the object of interest); by using a set of 'x' and 'y' calibrated wheels, then, the total area of the object can be determined [25]. Moreover, 'Z' axis calibration can be used to estimate the thickness of a microscopic specimen's. In modern systems such as LSCM, 'Z' axis is now usually associated to the collection of a series of images of various focal planes that can be, subsequently, used to construct 3D representations of the specimen. Sometimes, lower image stacks acquired by LSCM have lower voxel intensities when compared with the upper ones, i.e., there is intensity attenuation with depth. To reduce this, it is recommended to start the Z series at the deepest part of the specimen or, alternatively, it is also possible to correct this problem during image processing [25].

In the history of microscopes development, several devices were designed to assist the specimen's morphometry. To date, with the advances in microscopy (both in optical and fluorescent microscopy) and with image acquiring devices now coupled to microscopes, researchers have available a large offer of modern and efficient image analysis systems. At present, the most common technology used for microscope imaging are solid-state cameras (SSC) which have been used as acquiring devices: the detecting element itself consists of individual detectors (pixels) arranged in a square or rectangular array. Sensor chips can be produced to present millions individual sensors (pixels), each presenting identical features, such as response to light (gain and linearity) and, therefore, each individual pixel is viewed as a photometric sensor [19].

To use a SSC for photometry, the image that the individual detectors (pixels) see must meet the requirements of the Beer-Lambert Law, i.e., they must see a homogenous portion of the specimen. By calibrating the microscope lens system used, and deriving the area of the specimen in square microns seen by each pixel of the SSC chip, an appropriate magnification can be selected allowing accurate photometry. SSC are available in monochrome or color versions using two different technologies: three separate detector arrays and color cameras.

In three separate detector arrays (each presenting a color filter in front of the array), a prism or mirror system is used to split the image obtained into three separate but identical images. The filters are red, green and blue, since a red image, a green image and a blue image can be combined to create a full color image (RGB image). The major advantage is that every pixel is real. One disadvantage is there may be differences in sensitivity of a "red" pixel and a "green" pixel which are seeing the exact same spot of an image, requiring careful calibration and correction in the output between the separate detector chips.

Color cameras use a single detector and place a pattern of color dots over the individuals, again red, blue and green. Cameras used for microscope imaging are also described in terms of signal resolution per individual pixel (bit depth or gray levels): this specification describes the number of divisions of the signal between 0 (no signal) and 255 (maximum signal), corresponding to 256 levels [19,26].

Selection criteria for a new digital camera is complex and can be set according to: sensitivity, quantum efficiency, signal to noise ratio, spectral response, dynamic range capacity, and linearity, speed of image acquisition and readout, speed of response concerning changes in light intensity, geometry accuracy, resolution and adaptability to the microscope. Moreover, selection criteria must also include some experimental aspects related with the specimen wich are, in general, relevant: fixed specimens or live specimens; need of gray scale or true color images; sensitivity to low light levels (e.g. fluorescence); resolution; use in qualitative or quantitative applications. Most of these image acquiring devices are now connected to computers.

The use of computers became common to image analysis, particularly when analyzing large amounts of data or for quantitative information extraction. As camera resolution increases, they often exceed the display capability of many computer displays and, to avoid this, image should be reduced to fit within the monitor resolution. In these

circumstances, the displayed image may not accurately represent the real image that has been captured. An alternative is to divide the original images in small portions, each one keeping the same resolution as the real original image [19].

3.1 Imaging receptor immunostained specimens

Tissue sections were incubated with the primary antibody against the receptor in study, the resulting immuno-complexes detected with a biotinylated secondary antibody, and amplified by ABC complex, using 3,3 diaminobenzidine (DAB) as chromogen. Receptor DAB-immunostained processed sections should be imaged using brightfield optics on a light microscope in a given magnification (depending on the type of specimen/aim of the study) and individual images should be captured using a color digital camera (CCD charge coupled device) mounted on the microscope and connected to a computer. The illumination levels of the brightfield optics and camera exposure should be maintained at a given level throughout the investigation. The conversion of pixels to micrometers should be performed using a calibrated micrometer [27]. After captured and saving, each image file can then be converted to the appropriate color mode system accordingly to the type of specimen/aim of the study.

4. Image analysis

Independently of the quantitative method used, it is essential to consider the preparation of the specimens as a relevant issue: all specimens should be processed in the same way (specimen preparation procedure, light conditions and the microscope magnification) so that the quantification procedure will be reliable. Image analysis is currently defined as the extraction of meaningful information from images, mainly from digital images, by means of digital image processing techniques [28]. These techniques should be able to generate numeric data that, somehow, describe some particular aspect of a specimen, e.g. when the specimen is stained for a some specific constituent and, if the way how the label feature interacts with the tissue/cell constituent is known, it may be possible to use photometric's to produce numbers corresponding to the amount of the constituent present [19].

At present, software techniques for image analysis improved and computers became faster and more affordable. Several commercial image software packages are available for Windows and MacOS computers. There are also several freeware or shareware image analysis packages available at the internet such as, Image J (http//rsb.info.nih.gov/ij) [29]. Most of these software packages allow a variety of image analysis processes, but the terminology for specific types of processes differs between them. Although most image analysis software manufacturers provide technical support, in the internet we can find image analysis groups maintaining discussions that can be of assistance in case of troubleshooting/image analysis problems [19]. All image analysis programs must provide mechanisms to display images, read images from a source (camera or storage), and ultimately, save the image and any derived data. This allows the researcher to take advantage of the numerous offers for computer controlled image manipulation and contribute for results in less time and with higher reproducibility. Moreover, some of these software packages provide tools to make 2D and 3D reconstruction and visualization, image quantification, etc.

4.1. Color modes

The human eye perceives the outside world by receiving and processing electromagnetic radiation, wavelengths from 380-780 nm (visible spectrum) and the sensation of color results from different combinations of distinct light wavelengths. Nevertheless, there are considerable differences between images acquired using cameras connected to the microscope and the same image viewed by the human eye: a person with excellent eyesight, under ideal conditions, can distinguish between 30-35 levels compared to 256 or even more obtained from modern cameras. Indeed, SSC will detect intensity variations invisible to the human eye. For the computer, an image is just an array of values of the individual pixels (8-bits monochrome images, for example, corresponds to a sequence of numbers with values from 0 to 255). The display of colors on the computer monitor are usually defined in the RGB (red, green and blue) color mode, in a scale between 0 (corresponding to black) and 255 (corresponding to white) [19]. The split of RGB images into their red, green and blue components may be used to identify the component that yields the better contrast between pixels (clear delimitation). For example, in biological specimens stained with a chromogen such as DAB, the higher contrast is obtained in the blue component. When the contrast is not well defined between pixels in a particular component, it is mandatory to use simultaneously the three color components in order to perform a correct further image analysis [30]. The use of RGB mode is very simple: it can be displayed by using three separate electron guns to excite red, green and blue phosphors in the monitor screen. Images, then, are perceived by the human visual system as luminance, hue and saturation (all complex functions of the three color components) [31].

In addition to RGB mode, other color modes are available but those incorporating intensity and saturation information seem to be more intuitive to the human eye and are therefore more popular (an example is the HSI color mode since it closely resembles the human eye behavior [32]). For a given red, green and blue intensities of a pixel, the intensity (I) of the light source can be calculated:

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$$I= (R+B+G)/3^{i} \quad (1)$$

The saturation (S) is given, $S=(2/6^{1/2})(R^2+G^2+B^2-RG-GB-BR)^{1/2ii}$ (2)

Hue (H) can be expressed, $H=\tan^{-1}[3^{1/2}(G-B)/(2R-G-B)]^{iii}$ (3)

Some software includes circuits which promptly convert from RGB to HSI color mode or vice versa. This can be useful in certain applications. For example, in case of R=B=G, the S obtained is 0. Since this color space allows certain operations (such as luminance scaling and color shifting) to be performed with significantly less computational effort than that required if the same operations were performed in the RGB color mode.

4.2. Segmentation

Segmentation consists in the decomposition of the image in homogeneous regions in order to facilitate interpretation and should be one of the first steps to be carried out in image analysis process. In the last decade, several segmentation methods have been described and are available for the researcher use [33-35], however, the criteria of selection between these methods is complex. Indeed, it is not simple to evaluate the efficiency of the segmentation methods nor is the choice of a single method as the optimal one, to any use. Nevertheless, it is accepted that a successful segmentation requires segments to be homogeneous and statistically distinct from adjacent ones. In fact, if a segment is homogeneous, all putative fluctuations in pixel values will be purely due to speckle [36]. However, the majority of biological specimens are, generally, heterogeneous, which means that segmentation standard procedures will consider any visible heterogeneity as due to speckle. To avoid this, segmentation must be done in specimens that present clear delimitation between pixels (defined contrast). This can be achieved improving contrast and/or using particular features from the object of interest such as size, shape, density, brightness, texture, color, etc, since this specific feature allow a separation between the object of interest from the background. For example, by performing histograms in the individual components of the RGB images (red, green and blue components), it is possible to evaluate the contrast obtained for the image in study and select the component yielding the better contrast between pixels.

4.3. Image analysis processes

Several image analysis processes are available, some treat the image as series of pixels (point and area processes), other operate in the entire image (frame processes). A point process acts on an individual pixel within the image, and may modify the pixel value depending on the previous value. An example of point process application is the changing of the value of each pixel to some other value, depending on the original value. Such an operation makes use of a LUT (Look-Up-Table) and a common use is in a pseudo-color operation: where a gray scale image is divided into a number of "levels", meaning that all pixel values between 0-and 20 might be "colored" red, between 21-and 50 "colored" blue, etc. This operation might well make interpretation of a gray scale image easier [19] since the human eye can recognize color variations easier than density variations. In image analysis, the most usual application of point process is image thresholding. Thresholding is used to segment an image into areas that have some particular interest/feature, such as receptor staining pattern. The action is simple: the researcher selects a particular gray level (generally using the graphic tool available on the graphical user interface of the software package). This point process then sets all pixels with a value lower than this threshold level value to 0 and all values above this value to 1 (conversion to a binary image). While this simple threshold is sufficient for some purposes, the simple binary image is more commonly used as a "mask" to combine with the original image to produce an alternative image. A common implementation of this is the combination of the binary image in a way that all 0 or background levels are left 0 and all 1 pixels are left with their original value. Such an operation leaves the desired portion of the image visible while the remainder is eliminated from the image. A second thresholding from the opposite direction can be carried out in order to separate objects of medium gray from both lighter and darker ones [19,37]. For example, to segment specimens sections stained from those not stained, the researcher can define the minimum value in the gray scale (0 to 255) under which the object of interest (for instance: receptor staining pixels) is identified. This allows discriminating groups of pixels considered the object of interest from other groups of pixels (background). It is also possible to additionally combine the two threshold operations with a pseudo-color or LUT operation and place a transparent mask over the desired objects, leaving the entire original image visible.

Point processes can also be used to: i) change the overall intensity of an image: too bright images can be darkened by adding the same value to each pixel (i.e., assuming that 0 is black and 255 is white); ii) convert an image to the negative of the original image (the scale of black to white values is inverted: black = 255 instead of 0) and each pixel is mapped to this inverted scale; iii) the expansion of the gray levels in the image which can reveal/measure additional details [19,37]), i.e, image contrast stretching.

Area processes is a useful tool to derive information from the image or to alter the image in some specific manner by using groups of pixels. Usually, area processes involve a small portion of the image in a 2D matrix. The matrix is,

commonly, made up of an odd number of "row" pixels and "column" pixels (a convolution kernel). This explains why some area processes are named convolutions. Convolutions are, generally, based on 3, 5, 7 or larger dimensions matrix. A convolution matrix is defined and placed over the image and, each pixel over which the convolution mask lies, is multiplied by the number contained by the convolution mask. All these multiplied pixel values are then summed and value obtained is then used to replace the central pixel; the mask is moved one pixel further along and the process repeated [19,37].

In other such as frame processes, the operation use simple "Boolean operators" (AND, OR, XOR, NOT, etc) to add, subtract, multiply, divide or otherwise combine images to produce a new third image. For example, a common application of a frame operation is to correct a microscope image for uneven illumination [19,37]. Geometric processes are used to reconstruct or correct images, to rotate images, change scales, translate images and produce mirror images, to interpolate images from real size to a size that can be displayed on the PC monitor, etc. The operation move pixels within the image and can correct defects such as geometric distortions. Geometric distortion would include such image defects as a microscopic section that was attached to the slide in a manner that distorted the normal morphology. Geometric process could transform the image to straighten or otherwise return it to the shape believed to be correct [19,37]. Moreover, with microscopes systems such as LSCM being use to collect images from various focal plans, geometric processes have been used, for example, to align stacks of images or reconstruct 3D models of specimens, to create mosaic images, to reduce noise, etc.

4.4. Data treatment

After object segmentation, data is collected by integrating the: i) optical density , ii) size (area), iii) length of perimeter, iv) shape or other several forms of measurement related with the variation of intensity of pixels within the object. At present most image analysis programs offer interactive tools to measure image portions directly, e.g by drawing a line on the displayed image. Then, the data collected can be saved, statistically analyzed, graphically displayed and/or exported in formats compatible with common analysis tools, such as spreadsheets.

5. Automated and semi-automated computer assisted image analysis methods

It is increasingly true, for both routine diagnostic and research activities, that results are needed in shorter periods of time. The development of automatic systems based on modern software can, in most cases, overcome the classic quantitative methodologies inherent limitations but requires the use of a segmentation method appropriate to the specimen in analysis, an image process capable of extracting the object of interest from the background: automated and semi-automated quantitative computer assisted image analysis is suitable to generate profile measurements, facilitating the separation of the tissue elements or features of interest from background, using differences in size, shape, density, brightness, texture or color [13,27,38]. The criteria to choose the appropriate approach depend on the complexity of the specimen in analysis, the speed of both hardware and software in use and the validity of results.

Automated computer assisted image analysis reduces the variation of analysis of the staining levels of the sections under study, increasing data reproducibility [40] and can be successfully applied to analyze features in specimens that fill the entire microscope field. In spite of the undeniable capability of modern image analysis programs, sometimes, some manual interventions may be crucial and advantageous: semi-automated quantitative computer assisted image analysis procedures are influenced in one or more steps by the expert judgment, enabling segregation or segmentation of specimens based on particular features the computer is incapable to discriminate such as boundaries, layer types, etc. In such cases, the researcher evaluation is desirable to increase data accuracy in spite of a possible reduction in reproducibility (e.g. SACAIA method [13] and other methods applicable to DAB-immunostained specimens [27] or luminescence stained sections [41]). Additionally, there are limitations and potential drawbacks with automated computer image analysis procedures such as susceptibility to variations in staining [42-45]. By opposition, manual image analysis appears to be less vulnerable to variations in staining since human eyes present adaptive skills, justifying the requirements of specimen processing.

Computer image analysis extends the usefulness of immunostained sections by allowing extraction of 2D feature data such as feature count (number of stained structures), areas fractions (total area of features per unit of area of interest) orientation, shape and distribution. When properly adapted, it can also estimate data area density (number of features of interest per unit area) by using more refined methods of computation and standardization [41,46]. Nevertheless, to express the intensity of staining (epitope abundance), the performance of a reference standard curve is mandatory.

6. Image analysis of immunostained receptors: quantitative procedures

Quantitative information on the expression of receptors or other type of proteins/antigens can be combined with functional experiments results, contributing for an integrative view of receptors/proteins both in physiology and pharmacology. In spite of the importance of this subject few studies have addressed the extraction of quantitative

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information on the expression/presence of receptors [29,47-49], most of them by using quantitative flow cytometric procedures. It is also of major relevance to evaluate the validity of the new methodologies by comparing the data obtained with the new method with data obtained via classical methodologies. Semi-automated computer-assisted image analysis (SACAIA) has been previously validated [29] and has been used to quantify the presence of several receptors in different specimens (Table 1).

Images from tissues (arteries) section DAB-immunostained with the receptor antibody in study were assessed by quantitative histomorphometry: RGB images (three filters image) were opened and, the respective blue component, selected (the one presenting the best contrast); after the calibration step, the researcher must evaluate if the image requires some form of modification (for instance, evaluate if the vessel presents an irregular adventitia of connective tissue, which is particular difficult to be automatically recognized), and then, delimitate boundaries to clearly define the limit of the tissue section (specimen) in analysis, allowing the removal of the noise and the definition of the correct measurement area. After this step, the researcher should re-evaluate the image and decides whether to continue the process. This is of particular relevance in tissues that don't fill the entire image field, and therefore the delimitation of the boundaries aims at discarding any areas that might be incorrectly selected. At this point, if the image is OK, the user carries on the procedure; if not, it will be necessary to modify the image, until it is completely suitable for further steps. Afterwards, the user performs a thresholding operation in order to segment the image in both stained and non-stained tissue fraction areas.

The image can then be quantified by measuring the area of highlighted DAB-immunostained tissue (with the receptor antibody in study). Regions of intensity levels greater than the maximum threshold value, obtained for the control images (absence of the primary antibody against the receptor in study), are excluded and not considered as DAB-immunostained. The corresponding areas of stained tissue are temporary saved in a data file and all the entire procedure is repeated again, opening the next image. At the end, when all images have been quantified, the user saves the results in a permanent storage data file and the operation is completed.

To correctly segment our object of interest we used control specimens to clearly define the limit value (threshold value) in the color scale (0-255) under which the object of interest (stained pixels) can be identified (Table 1). Therefore, we must guarantee that images from control tissues sections are acquired in the same microscope/CCD camera operating conditions as the DAB-immunostained tissue sections with the receptor antibody in study.

Ti = DAB-stained tissue area + non-DAB-stained tissue area (4)

Where DAB-stained tissue area (Si for the field i); Ti is total tissue area in each image field of area (Fi). To measure the level of immunohistochemical staining (Sf), the fraction of the tissue stained with DAB was quantified:

$$Sf = Si/\Sigma Ti \quad (5)$$

Where Sf is the staining fraction area, Si is the stained tissue area in each image field, and Ti is the total tissue area in each image field. Note that Ti is not equal to the area of the image field in tissue samples that do not fill the entire image field of the microscope.

Table 1. Quantification of rat vascular tissue receptor antibodies staining fraction areas

Receptor	Abdominal aorta	Mesenteric artery	Tail artery
	Staining fraction area Mean±SEM (*n*)	Staining fraction area Mean±SEM (*n*)	Staining fraction area Mean±SEM (*n*)
Adenosine A_1	72.7±3.1 (34)	34.6±2.3 (6)	88.1±5.0 (13)
Adenosine A_{2A}	74.0±2.9 (38)	-	89.6±4.6 (15)
Adenosine A_{2B}	83.3±2.9 (39)	-	79.3±4.6 (15)
Adenosine A_3	34.9±3.4 (28)	-	0.31±4.6 (15)
Angiotensin AT1	-	75.7±4.8 (9)	-
Angiotensin AT2	-	45.8±5.4 (9)	-

Results obtained using SACAIA method [29]. Results are expressed as mean±SEM; *n* represents the number of tissue sections quantified.

In summary, SACAIA method is, as others studies [27,46,50,51], based on quantitative routines to computerized image analysis of immunostained specimens using DAB as a chromogen and, additionally, is applicable to both specimens occupying completely the entire microscope field, such as large size specimens (e.g. tumor biopsies) or using high magnification to study the object of interest, and specimens that did not fill the entire microscope field (for instance, at a lower magnification) and accurately determine our object of interest, or to specimens where a particular layer should be accurately defined. Indeed, to convert brightfield color images of DAB-immuostained antigens (such as

receptors) into their blue component (the one presenting the best contrast) and to extract the object of interest, methodological procedures can be customized according to the type of specimen in analysis. In this sense, it is possible to use automated or semiautomated assisted image analysis to quantify one or more receptors in tissue sections. Nevertheless, according to the specificities of the immunohistochemistry used, adjustments to the quantification methodology may be required, resulting in the customization of some of the procedures. In fact diverse chromogens and/or counterstains can be used determining differences in the contrast within the RGB components and/or in segmentation and, therefore, appropriate adjustments are recommended. Moreover, we should never forget that in spite of these quantitative analysis methodologies are of indubitably usefulness, both in basic and clinical research, although they allow the researcher to achieve higher quality of data, the complete procedure of image analysis still needs to be validated. Only through an adequate and criterious validation it is possible to ensure that the application of the methodology is unbiased, credible and reliable.

Reliable quantitative data related with the expression of receptors (or other type of proteins) can be precious and open possibilities combined with functional data to new perspectives of study, contributing for an integrative view of receptors/proteins, both in physiology and pharmacology.

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