Computer-aided techniques for Chromogenic Immunohistochemistry: Status and Directions

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Abstract *Although immunohistochemistry (IHC) is a popular imaging technique, the quantitative analysis of IHC images via computer-aided methods is an emerging field that is gaining more and more importance thanks to the new developments in digital high-throughput scanners. In this paper, we discuss the main steps of IHC and review the techniques for computer-aided chromogenic IHC analysis, including methods to determine the location of interest of the antigens and quantify their activations. Moreover, we discuss the issues arising from the standardization of the immunostaining process, that are generally overlooked by current literature, and finally provide requirements for reliable computer-aided IHC quantification.*

1 Introduction

The continuous developments in bioimaging technologies (especially microscopy) have established the ultimate proliferation of computer-aided biological imaging techniques as an effective way of extracting clinical and functional information from molecules and tissues [1] [2]. Pathologists are relying more and more on microscopy image analysis to assess the presence and activity of target antigens in the tissues, with important applications in the diagnosis and assessment of tumors as well as for several research purposes.

The analysis in situ of the activation of specific proteins provides critical information about multifactorial genetic pathologies and tumors [3] [4], supports the design of personalized targeted therapies [5], allows to define a group of potential candidates to protein family-inhibiting therapies [3] [6] [7].

One of the most popular imaging techniques in this field is immunohistochemistry (IHC), that uses marked antibodies to link specific proteins in situ, as well as their ligands; the evaluation of the colored stains at the specific sub-cellular regions where the markers are localized (i.e. nucleus, cellular membrane, cytoplasm) provides information about the presence and the activation of the target proteins in the tissue, and therefore it is useful for the assessment of important pathologies [3] [4]. Immunohistochemistry is widely used in clinical and research laboratories since the early seventies for the qualitative assessment of the tissue specimens, and it has acquired a central role in pathology thanks to its wide availability, low cost, easy and long preservation of the stained slides [8]. In the last few years, with the continuing developments in digital, high-throughput tissue slide scanners, IHC is gaining more and more importance as a technique able to provide not just qualitative but also semi-quantitative or quantitative measurements of protein activations. Nevertheless, this shift from qualitative to quantitative raises a lot of issues about the robustness of the IHC assay; moreover the reliability of the results obtained through visual evaluation of the specimens, inherently subjective, is heavily questioned [9].

The automation of the image analysis task through computer-aided techniques is acknowledged for being a possible solution towards the standardization of the IHC test and the extraction of reliable quantitative measurements of protein activation [10] [11] [12]. On top of that, the new demands of modern pathology and personalized medicine require precise and highly localized measures of protein activations, at cellular and sub-cellular level [11], which is not feasible with simple visual evaluation. This has determined a growing effort in the development of automated techniques for the segmentation and the analysis of IHC tissue images, able to recognize and measure the antigens' activations within their specific regions of interest [13].

Different tissue images associated with different diseases may exhibit unique characteristics, both in terms of tissue morphology and evaluation procedure, demanding specific image analysis pipeline. However, several image analysis components remain common in most of the applications, so that it is possible to identify a typical work-flow for computer-aided IHC analysis. This work-flow contains sequential segmentation steps with the aim of identifying the specific regions of interest of the studied antigens, followed by the quantification of the antigens'activation.

After introducing the background of digital pathology and immunohistochemical analysis, in this paper we provide a critical overview of the image analysis techniques applied to the main steps of IHC, analysing potentials and limitations of the different approaches, and we discuss the open challenges for a standardized quantification of protein expression.

2 Digital microscopy image analysis

Digital microscopy is the effective integration of digital imaging and light microscopy, where a comprehensive platform combining optical, electronic, mechanical, image processing and computer technologies assists the pathologist in acquiring, observing, analysing and sharing pathology image data in digital form [14]. In the past few years, the digital revolution in the field of bioimaging has rapidly

transformed the work of the pathologists, traditionally limited to the microscopic observation of the samples, into a completely digital work-flow. This has determined the definite rise of quantitative over qualitative analysis of the images.

Main categories of digital microscopy analysis can be identified according to their object of study, or either according to the technologies that are employed to acquire and analyze the images.

A first categorization distinguishes digital pathology into cytology, that is the study of isolated cells or cells clusters, and histopathology, that is the microscopic evaluations of pathological tissue sections, where different tissue components are dyed with two or more stains. Traditionally, the most widely used dyes for histological analysis are Hematoxylin and Eosin (H&E), where H stains nuclei blue and E stains cytoplasm and connective tissue pink [15]. Another widespread technique in histopathology is Immunohistochemistry (IHC), that uses labelled antibodies to localize specific activations of antigens or proteins in the tissue sections.

A second categorization distinguishes digital pathology images based on the type of microscopy that is employed for image acquisition. Together with classic light microscopy, several image modalities have been established, including fluorescence and immunofluorescence microscopy, confocal microscopy, multispectral microscopy and electron microscopy [15] [16]. All these modalities have seen huge technological advancements in the last few years, with the main consequence being a real explosion of number and density of image data.

The technological advancement is accompanied by the proliferation of more and more sophisticated methods for analyzing this massive quantity of image data: comprehensive CAD systems for digital pathology have been recently proposed, with analysis modules including preprocessing, image segmentation, feature extraction, classification and postprocessing (see [15] and [16] for reviews). Most of the image analysis methods can be applied with minor modifications to different types of images, while others (for example, spectral deconvolution techniques [17] [13]) are taylored to a specific imaging technology. As different imaging technologies extract different information from the tissue sections, a recent challenge of computer-aided analysis is also the effective integration of these data to provide more insights about the pathological process. As such, the research on multimodal data fusion has been very active, including H&E and IHC slide registration [18][19].

While digital pathology offers very interesting and dense information, the correct analysis and interpretation of these data is still an open problem, especially in the field of IHC. In the next sections of this paper we will provide more insights about this problem with special regards to chromogenic IHC, that is the most widely diffused in standard laboratories and addressed by literature. Fluorescence microscopy and multiple labelling strategies are, on the other hand, increasing in popularity and will rapidly emerge as a prime alternative to chromogenic approaches. More details about these techniques can be found in [15] and [16].

3 Immunohistochemistry: fundamentals

Immunohistochemistry (or IHC) is a widespread procedure in digital pathology that refers to the process of localizing antigens (e.g. proteins) in the tissue; the localization is obtained exploiting the principle of antibodies binding specifically to antigens [20]: the tissue is stained with the labeled antibodies that selectively bind to the antigens under investigation.

Immunohistochemical staining is widely used in the diagnosis of abnormal cells such as those found in tumors. Specific molecular markers are characteristic of particular cellular events such as proliferation or cell death (apoptosis). IHC is also widely used in basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a tissue [20].

The visualization of antibody-antigen interactions can be accomplished in a number of ways and methods based on the type of tissue under investigation and on the degree of sensitivity that is required by the specific application. In the most common instance, an antibody is conjugated to an enzyme, such as peroxidase, that can catalyse a colour-producing reaction; alternatively, the antibody can also

be tagged to a fluorophore, such as fluorescein or rhodamine.

The typical IHC work-flow prior to microscope evaluation consists of two main steps, namely preparation of the sample and immunostaining (see Fig. 1); each of them implies a complex sequence of mechanical and chemical procedures having a major impact on the quality and processability of the IHC images and on the repeatability of IHC quantifications. In this work, we provide an overview of the typical IHC work-flow and discuss the problems related to the standardization of its main steps.

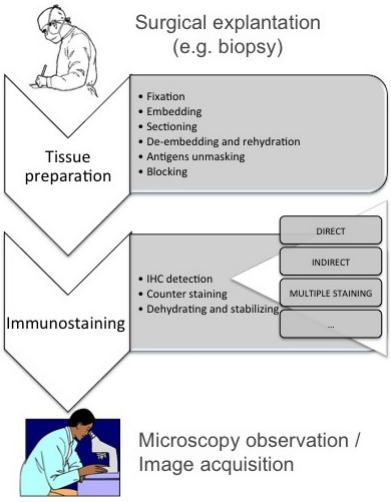

Figure 1: Main steps of a typical IHC procedure [20].

In the classical protocols, after surgical explantation the tissue specimen is immersed in a fixative in order to ensure the preservation of tissue architecture and cell morphology. Aldehydes are the most commonly used fixatives in routine histological laboratories, including formaldehyde (either derived from formalin or depolymerization of paraformaldehyde), glutaraldehyde or a mixture of the two [21]. After fixation the specimen is usually embedded in a material with similar mechanical properties (e.g. paraffin or plastic resin) and sectioned in thin slices by means of a microtome. Pre-embedding with agar or agarose, routinely performed to encapsulate cells from cell suspension, can be even useful with tissues in order to achieve the right orientation and position of the samples and to protect them from drying and wounding [21].

Some antigens would not survive aldehyde fixation and paraffin embedding: in this case, tissues can be rapidly fresh frozen and undergo cryosectioning. Cryosectioning has some disadvantages over paraffin or plastic embedding, including poor morphology of the tissues, poor resolution at higher magnifications and need of special storage for the specimens. Another alternate technique is vibratome sectioning, that can be applied either to fixed or unfixed tissues and does not require embedding of tissue in wax or resin. It has the advantage of preserving 3D structures well, but the sectioning process is slow and difficult with soft and poorly fixed tissues, and might produce artifacts in the sections in the form of chatter marks or vibratome lines [22].

Vibratome sectioning is able to produce thick tissue sections (>20 μm) which can be imaged to reveal the structure of the underlying tissues. This is very useful in many applications, such as the assessment of bio-compatibility of implanted devices [23]. Nevertheless, the penetration capability of standard optical technologies is generally limited, placing severe constraints on the maximum thickness of the tissue sections that have to be imaged. Accordingly, the largest proportion of material for immunohistochemistry is in thin slices (3 to 10 μm). Recent works propose methods able to overcome this limitation leveraging on non-standard microscopy techniques such as optical projection tomography (OPT) [24], or using aqueous reagents to render the samples optically transparent [25]. These techniques can image samples with a thickness in the range of several millimeters.

After the sectioning, the sample is de-embedded and optionally treated with antigen retrieval reagents in order to enhance the antibody-antigen binding, that might be inhibited due to the fixation and paraffinization process. Incubation with blocking agents (e.g. serum proteins) prevents non-specific binding of the primary antibody.

Staining methods can either be direct or indirect [20]. Direct method, rarely used due to very little signal amplification, involves the incubation with only one labelled antibody reacting directly with the antigen in the tissue sections, whereas indirect method involves a first layer of unlabelled primary antibody reacting with the tissue antigen, and a second layer of labelled secondary antibody reacting with primary antibody. Compared to direct method, indirect staining method shows a higher sensitivity due to signal amplification through several secondary antibody reactions with different antigenic sites on the primary antibody.

The labelling of the secondary antibody is obtained with a fluorescent dye (i.e. indirect immunofluorescence method) or either with an enzyme such as peroxidase, alkaline phosphatase or glucose oxidase (i.e. indirect immunoenzyme method). The enzyme in turn reacts with a chromogen such as DAB (3,3'-Diaminobenzidine), which produces an intense brown staining that localizes the primary-secondary antibody reaction. Optionally, a counter staining can be accomplished with a contrasting colour to highlight the components that are not made visible by the principal stain (e.g. hematoxilin counter staining, responsible for the blue colour of nuclei in H-DAB stained specimens). Other typical staining protocols other than H-DAB include staining with Haematoxylin and Eosin (H&E), Feulgen Light Green, Giemsa, Fast Red-Fast Blue-DAB, Methyl green and DAB, Haematoxylin, Eosin and DAB (H&E DAB), Haematoxylin and AEC (H AEC), Azan-Mallory, etc.

After being stained, the specimens are mounted to be observed on the microscope (see Fig. 1). Only when using hydrophobic mounting media, the specimens need to be pre-emptively dehydrated in alcohols. More details about the preparation of the samples are provided in [20].

Methods and protocols used to prepare the specimen introduce a series of critical sources of variability (e.g. type and duration of fixation, consistency and thickness of the sections, temperatures, pH, enzyme concentration and incubation times), affecting the morphology of the tissue, the intensity of the stains, as well as the number of cells demonstrating a positive staining reaction. Therefore all these variables need to be standardized and controlled accurately in order to assure the consistency of IHC evaluation.

In the last few years many efforts have been made towards an improved control of the overall procedure; moreover, nowadays the market offers highly specialized programmable systems that are able to handle the main steps of the process - from embedding and sectioning of the specimen to antibodies incubation and immunostaining - in a semi-automated or fully-automated way: this minimizes dramatically the sources of variability that affect the quality of the staining, giving boost to the rising of standardized quantitative IHC [26].

4 The standardization issue and the role of automated image analysis

The need for standardization in IHC has been stressed as a major critical issue since the late 70s. In fact for a long time this widespread technique, despite being extensively used for either diagnostic and research purposes, has been relegated to a secondary role because of the extreme variability of the results. This variability covers all the aspects of the assay, from sample preparation to image analysis.

The most important contributions to IHC standardization come from the works of Clive R. Taylor, which published a number of papers addressing this issue. In particular, in 1992 he proposed the so-called total-test approach [27], a comprehensive procedure covering for the first time all the major aspects of the immunohistochemical protocol and lately summarized the main requirements for IHC standardization in three major points [11] [28]:

1. follow staining protocols carefully avoiding any possible source of variability (e.g. staining con-

ditions, timing, storage, etc.);

2. validate experimentally every new reagent which may represent an unknown source of variation in the IHC assay;

3. use positive-control tissues, fixed and processed at the very same way as the specimens under investigation, to validate each reagent and protocol.

The latter point is particularly critical for quantitative IHC, as positive controls can provide information about the quantity of analyte per specific cell types, before and after fixation treatments, and may serve as calibration points for the antigen's quantification.

In the last decade many attempts have been made to introduce reliable positive control tissues, including cell line standards processed simultaneously with the specimen (e.g. Quicgel method [29]), short constrained peptides used as antibody targets directly to the glass slide [30], protein-embedding techniques [31] as well as IHC standardization through Antigen Retrieval [28]. More recent works even proposed internal controls consisting in tissue components that are present in the same tissue section as the target antigen when tested by IHC [32], or either in the same tissue microarray [33].

As quantitative IHC implies to measure the target antigen in comparison with a reliable positive standard serving as a reference [28], a ever-increasing number of investigators believe that computer-aided analysis is the obvious answer to IHC standardization [34] [35] [36] [37] [12]. In fact, digital image analysis allows to quantify antigen's activation either from the specimens and the controls directly from the features of the digitalized images acquired in the same conditions; it follows that in principle computer-aided analysis is able to avert the variability and subjectivity that traditionally affect IHC.

On the other hand, computer-assisted image analysis relies on the calibration and standardization of the immunostaining process, and therefore needs to be structurally integrated into a controlled and reproducible immunohistochemical assay (see Fig. 2). The reason is twofold: firstly, because a reproducible immunostaining process decreases the sources of variability that affect the quality and the processability of the digitalized images; secondly, because the positive controls can be used to assess the relation between antigen activation and the features measured on the digitalized images, serving as calibration points.

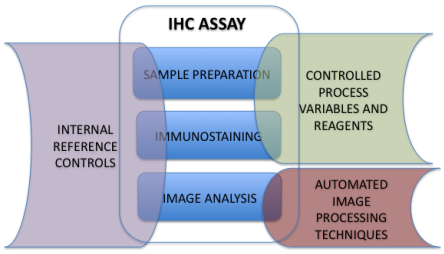

Figure 2: Main requirements for standardization, covering all aspects of the IHC assay.

It follows that standardized quantitative IHC and computerized image analysis are strongly and unavoidably interconnected, as a natural evolution of the "total test approach" proposed by Clive R. Taylor in 1992. As reported in Fig. 2, the ideal IHC assay leverages on three major solutions: (i) internal reference controls processed simultaneously with the specimen, to calibrate the entire IHC work-flow; (ii) standardized and controlled IHC protocols, to assure the quality, processability and repeatability of the IHC images; (iii) automated image analysis techniques, to provide repeatable quantitative measures of the biological phenomena of interest.

The primary role of automated image analysis has been recently strengthened by the growing demand for cell and sub-cell specific analysis. In fact, as recently acknowledged by modern pathology, for maximum significance immunohistochemistry should address per cell rather than per tissue analysis

of the target proteins: in fact, cells have been ultimately recognized as the fundamental units of behaviour in multiple molecular pathways at the basis of pathology and cancer biology, so that the relevant metric in cancer development relies on their specific individual phenotypes [11]. This implies that the analytes should not be assessed on average in the whole tissue but in identifiable individual cells, which is definitely not compatible with traditional visual evaluation. For this purpose, there is a growing demand for automated image processing techniques able to identify cell by cell the specific location of interest of the studied receptors.

5 Computer aided IHC analysis

With the growing awareness that computer-assisted technologies are the best solution to reduce the variability of pathologists evaluation and provide highly specific per-cell information, the market nowadays provides a good number of image analysis systems for IHC. The availability of such systems in pathology laboratories, very limited at the beginning due to high costs of acquisition and maintenance, is now starting to become wider especially in the US thanks to the intervention of medical association, that are pushing for the use of image analysis tools that can help the pathologists with the accuracy and consistency of their IHC results [38] [39]. The American Medical Association (AMA) even created a specific reimbursement code for such computer-aided systems, namely CPT 88361.

Different categories of IHC image analysis may apply specific pipelines to obtain different outcomes. Nevertheless, a typical work-flow of computer-aided IHC analysis can be identified, containing a sequence of image analysis modules that apply to most of the categories. This work-flow is shown in Fig. 3. On the left of the work-flow, we report a list of main categories of IHC analysis, including the study of cell proliferation or angiogenesis for tumor assessment, the analysis of tissue composition, the detection of rare events, and multi-stain quantification; on the right we report a few examples of quantitative outputs obtained applying each category of computer-aided analysis.

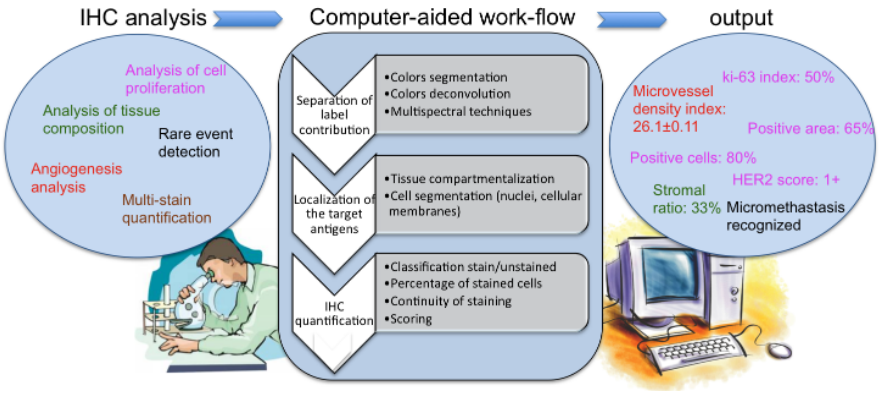

Figure 3: Typical work-flow of computer-aided IHC analysis. On the left, main categories of IHC image analysis; on the right, examples of correspondent quantitative outputs.

First of all, the input image is separated via hardware or software into the separate contributions of the different labels, so that the specific information carried by each colored dye can be evaluated and processed separately; in applications that require multi-stain quantifications, this module allows to distinguish and measure the contributions of several antigens in the tissue. The approach depends on the imaging technology.

Tissue compartmentalization is then generally needed in order to determine tissue composition (e.g. for therapy assessment or in-situ characterization of implanted devices), or either to limit the region of interest to the only parts of the specimen that are occupied by the antigen of interest, disregarding non interesting areas such as stroma and blood vessels. Mostly used techniques for tissue

compartmentalization are generally based on classification or clustering of features extracted from the image (either morphological or color-based features). Tissue classification/clustering and pattern recognition techniques are also used for the detection of rare events such as micromethastasis or the recognition of microvessels in tumor angiogenesis analysis.

The following step after tissue compartmentalization is cell segmentation, i.e. the identification of the sub-cellular region that is targeted by the studied antigen. This is important because different antigens are expressed in different portions of the cell, such as the nucleus, the cellular membrane or the cytoplasm (see Fig. 4); since the interested areas may be very little compared to the overall area of the cell, the analysis needs to be restricted to the sub-cellular portion of the cell that is targeted by the antigen. For example, membrane protein activity can be quantified on the base of the membrane stain continuity, in terms of rate of stained membrane pixels [40]. This requires to delineate the whole cellular membrane and then to differentiate between its stained and unstained pixels. Other antigens may be activated in the nuclei, or in the cytoplasm of the cell: this calls for techniques able to provide fully-automated segmentations of all the compartments of the cells. Segmentation approaches may vary from ad-hoc solutions to the most popular Computer Vision techniques such as watersheds, active contours, multiscale analysis and Markov random fields. More details will be provided in the next sections.

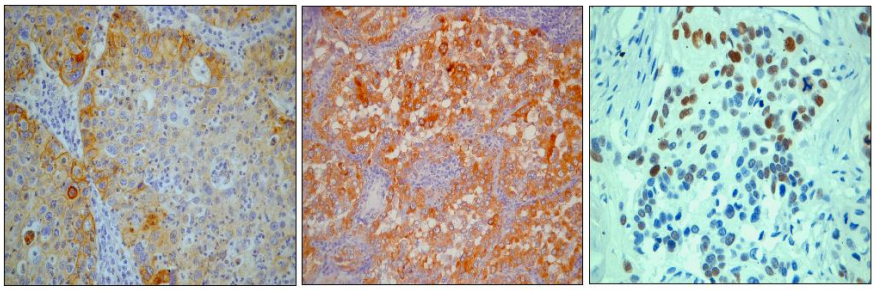

Figure 4: H-DAB images of lung tissue, respectively with membrane, cytoplasm and nuclear activations. The brown dye (DAB) highlights positive activations of the antigen; the blue dye (H) is used for counterstaining.

The intrinsic complexity and variability of IHC tissue images make the segmentation task particularly challenging. Sources of noise and variability may arise from the sample preparation and immunostaining process, as well as from the pathological process itself, with unpredictable effects on cells' morphology and proliferation.

Summarizing, the main features of IHC images that are responsible for making the segmentation challenging are the following:

1. The presence in the image of tissue components that may be not of interest for the analysis (e.g. lymphoids, blood vessels, stroma, etc.), representing a major source of noise for the quantification.
2. The superposition of different tissue slices, due to the unidealities of the sample preparation process; this superposition may generate considerable artifacts such as incomplete or clustered cells.
3. The colocalization of different stains, resulting in a poor separation of the contribution of the dyes.
4. The presence of clustered or touching cells.
5. The non-predictable morphological variations of the tissue induced by the pathology.

6. The lack of signal coming from inactivated portions of the cells, that interfere with the segmentation process (e.g. in case of membrane protein staining, as discussed in Section 7.3).

7. The heterogeneity (in terms of both intensity gradient and color) of the regions to be recognized. This is mainly due to uneven activation, dyes superposition or variation of illumination.

These heterogeneities are partly intrinsic of IHC technique, and therefore unavoidable. Nevertheless, major improvements in the quality and processability of the images can be achieved with the standardization of IHC assay [11]. In particular, the imposition of well-defined standards for the preparation of the specimens (e.g. type and duration of fixation, consistency and thickness of the sections, etc.) leads to a better consolidation of image features such as stains' intensity and the localization, definitely serving the purpose of tissue and cell segmentation.

After the specific locations of the antigens have been determined by the segmentation techniques, it is possible to extract the quantitative parameters from the images, which may vary depending on the disease or the application (see Fig. 3 for a few examples). Widely used quantification approaches include counting of stained cells, calculation of the stained areas and the evaluation of the staining continuity. The extracted parameters can also be used to classify the specimens into a set of pre-defined classes or scores of positivity.

Thanks to the ever-growing popularity of computer-aided IHC, commercial microscopy software are nowadays available covering most of the work-flow steps [41] [26]. The available computer-aided solutions usually allow the user to select the sub-cellular location of interest for protein activity quantification in a semi-automated or automated way; nevertheless, these tools are based on simple approaches that suffer due over-generalization, and most of the times require extensive user interaction, so that the objectivity of the result tends to be lost [8] [10] [41]. In particular, a number of commercial products require the user to select manually the areas that are richest in the cells targeted by the studied antigen [42] [43], to set intensity thresholds or levels to distinguish the cellular patterns from the background [44] [45] or to outline a set of representative cells [46] [47].

As a consequence of that, the demand for IHC automatization, especially related to the steps of tissue compartmentalization and cell segmentation, is not entirely addressed by the available commercial solutions, which opens the field to extensive and continuous research in this area.

In the following sections the main approaches for computer-aided IHC analysis are reviewed, covering the main steps of the work-flow shown in Fig. 3.

6 Separation of label contributions

The first step after image acquisition and digitalization is the separation of the contributions of the different labels (see Fig. 3). In this phase the contributions of the main chromogen (labelling the test antigen) and of the counter stain are distinguished so that their specific information can be processed separately.

Specific spectral deconvolution and unmixing approaches are applied in case of multispectral microscopy images [17] [13]. Multispectral imaging is an advanced imaging technique that acquires images where each pixel contains a spectrum (intensity as a function of wavelength) of the collected light at that location, rather than the typical 3 data points (red, green and blue) found in RGB images. These solutions are generally hardware-based, and will not be addressed by this survey.

Multi-channel fluorescence microscopy [48] allows to acquire the stains information already on separate channels. Even in this case color separation of fluorescence emission is achieved in hardware by using a set of optical bandpass filters that select different parts of the emission signals for every image channel [48].

Most of the IHC analysis in the standard clinical practice are usually performed through conventional light microscopy and digital RGB cameras, so that specific software solutions are needed to separate the stains' contributions directly from the RGB image. Literature reports several approaches including colors classification methods, that associate each pixel of the image to a different stain

based on thresholds in the HSI (Hue-Saturation-Intensity) space [49], as well as color deconvolution methods based on geometrical transformation from RGB space to the chromogen and counterstain space [50]. The latter approach has been demonstrated to yield to better stains' separation in presence of colocalization or spectral superposition of the dyes (e.g. for H-DAB stained images).

7 Selection of the location of the target antigen

Several segmentation and image classification techniques are applied to the aim of selecting regions of interest in the images, depending on the specific tissue or cell locations targeted by the studied antigens (see Fig. 3). These techniques can be categorized based on the anatomical region of interest they are target at. Tissue compartmentalization is generally aimed at classifying the specimen into two or more broad tissue areas, while cell segmentation techniques identify the main sub-cellular compartments of the cells. These techniques are reviewed and discussed in the following subsections.

7.1 Tissue Compartmentalization

The compartmentalization of different types of tissue is a critical step for histopathology, with aims including the study of tissue composition, the identification of cancerous vs. normal tissue areas, the identification of tissues of interest in the specimen, or the categorization of the tissue into several prognostic groups.

Human visual representation and recognition system is inherently object-based, as opposed to the pixel-based representation of the digital images [51]. As such, humans tend to understand and describe images in terms of abstract objects, and the human brain categorizes the images on the base of these high-level abstractions. This high-level representation is renowned for being extremely efficient with respect to the recognition and classification task, comparing to exhaustive pixel-based representation. Therefore, image processing methods try to mimic human-like object level representation of the images in several applications, including the task of tissue segmentation in histopathological imaging. The process of extracting such object level features from the images is universally known as *feature extraction*.

The majority of image features in the field of tissue classification are based on tissue morphology [52], [53], including area of the tissue, elongation, eccentricity, feret diameter, etc. Another important set of features commonly used in tissue classification are based on texture [54] [55], [56] which is generally defined as complex visual instances composed of entities, or patterns. These patterns show characteristics of brightness, color, shape, scale, etc. with perceived properties such as uniformity, roughness, regularity, periodicity, frequency, phase, directionality, coarseness, randomness, fineness, smoothness, etc. Texture analysis is a widespread methodology in medical image analysis, especially in histopathological image analysis, due to the highly textured appearance of cellular and extra-cellular structures.

Another set of features that are less extensively used but useful in some tissue classification applications are spatially related (i.e, topological) features. They are based on graph theoretical representation modelling different tissue components as well as their spatial arrangement [57].

After feature extraction, the tissue recognition/classification step is usually exploited. The most widely used approaches can be roughly categorized in two main groups:

1. supervised approaches, where the output is inferred through machine learning techniques (e.g. active contours, Bayesian learning, kernel-based techniques [58]) by a pre-labelled set of training instances; this requires the learning algorithm to generalize from the training data to unseen situations in a "reasonable" way.

2. unsupervised approaches, where the learning algorithm is given only unlabelled examples, and needs to extrapolate the output from the data characteristics [59].

Among the supervised techniques, some of the most popular approaches are Bayesian learning [56] and Suppport Vector Machines (SVM) [60] [61]. Unsupervised approaches, on the other side, include Self Organizing Maps (SOM) [62], hierarchical clustering [63], as well as k-means clustering [64]. The most suited approach needs to be chosen based on the morphological/textural characteristics of the tissues that have to be compartmentalized. The unsupervised methods are generally to be preferred when the features of the tissues may considerably vary from specimen to specimen [65].

7.2 Segmentation of nuclei

The accurate segmentation of the nuclei is one of the most important steps for quantitative IHC image analysis. Characteristics of the nuclei such as nuclear size, poleomorphism and chromatic appearance and texture have been extensively validated as prognostic markers of tissue malignancy in several cytological applications [66]. The assessment of nuclear antigens such as the fraction of Ki-67-positive tumor cells (the Ki-67 labelling index) is often correlated with the clinical course of cancer. The best-studied examples in this context are carcinomas of the lung, prostate, brain and the breast, where the prognostic value for survival and tumor recurrence have been repeatedly proven in uni- as well as multivariate analysis [67].

On the other hand, nuclear segmentation has been proven to be important even in applications aimed at assessing membrane and cytoplasmic markers and features. In fact, the nuclei are the most used spatial reference for the segmentation of the other cellular compartments and the whole cell in general. Therefore the accuracy of nuclear segmentation often reflects on the accuracy of the delineation of other parts of the cell and on the quality of IHC quantification in general.

The simplest approach for segmenting nuclei is a global thresholding [68], where a single intensity threshold is used to distinguish the nuclei from the background. The value of the threshold can be adjusted manually or either determined based on image histogram [69] [70]. Such methods work reasonably well in high-contrast images, but they are not suitable at all for most of IHC tissue applications, showing varying features and inhomogeneous illumination. Adaptive local thresholding techniques, which utilize local content information to separate background from foreground, produce significantly better results in this context [65].

Other popular approaches for nuclear detection are flooding algorithms such as watersheds [71], that can be effectively used to separate clustered nuclei [72] [73] [74] [75]. A major drawback of these techniques is that they often result in over-segmentations and multiple watershed lines, especially in presence of texture or staining inhomogeneities. On the other hand, the attempt to overcome over-segmentation through seeded watersheds [75] presents problems in obtaining only one seed per object and often resorts to extensive user-interaction.

Widely used in nuclear segmentation methods are also edge-based techniques (e.g. Canny [76] [77]) and active contours [78] [79] [80] [81] [82] [83] [84] [72]; nevertheless, these approaches suffer due to remarkable intensity variations within as well as outwards nuclei. In particular, active contours performance [78] [79] [80] [81] [82] [83] [84] [72] is highly impacted by the noise conditions and the massive presence of foreign particles which may deviate the initial curve from the target boundary. Edge-based techniques include also multi-scale analysis [85], that can handle intensity variations but is led to errors by overlapping nuclei with weak edge information.

Another category of nuclear segmentation techniques based on geometrical templates or on fixed models of the tissue morphology, such as in [86] [87], suffer due over generalization because of the non predictable shape and size variations of the cells; these variations are induced by the pathology as well as by the mechanical and thermal stress related to the preparation of the sample.

Alternative approaches include graph-cuts methods [88] [13] and Markov random fields [90], that on the other hand often suffer due under-segmentation. Graph-cut methods have the disadvantage of requiring good initialization, therefore they are most effective when used in combination with other methods. For example, [89] proposed a hybrid approach where the initial segmentation is obtained with binarization based on multi-scale Laplacian of Gaussian filtering and subsequently refined using the graph-cuts algorithm.

In conclusion, nuclear segmentation is still a challenging task, so that one single approach is not sufficient to obtain satisfactory results; therefore the most successful segmentation techniques are rather a pipeline or combination of several approaches, where several segmentation techniques are tailored to the specific issues of IHC images. Most of the proposed pipelines apply local or adaptive binarization to distinguish the nuclei from the background, improved watersheds techniques to separate clustered nuclei, and either active contours, graph cuts or extensive morphological post-processing to regularize the shapes and refine the nuclear boundaries [65] [91] [89]. Nevertheless, manual intervention is still necessary to set-up the parameters of the segmentation or to resolve the most difficult/ambiguous cases.

7.3 Segmentation of cellular membranes

The cell-by-cell segmentation of cellular membranes in immunostained tissues is one of the most critical tasks in the work-flow of computer-aided IHC, especially in chromogenic images. The most challenging issue is related to the reconstruction of the membranes in the portions that are negative to the target receptors (i.e. where the target antigen is not present), that are not revealed by the stain and then not visible.

The lack of intensity or gradient magnitude variations in the unstained parts of the membranes as well as the staining heterogeneity that is instrinsic of IHC imaging invalidate segmentation methods detecting intensity or gradient variations between the background and the pattern to be segmented [73]. Active contours approaches [78] [79] [80] on the other hand overcome the problem of connecting broken contour lines by modelling the target pattern with a closed curve; however they are in general extremely sensitive to initialization as well as to staining artifacts which may attract them far from the target membrane.

The largest amount of literature in the field of cellular membrane segmentation addresses fluorescence or confocal microscopy images, where most of the challenges are related to the non-uniformity of the fluorescence signal, which may create variations and gaps in the membrane continuity. Interesting approaches have been proposed in this field: [92] presented a method based on Voronoi regions with a metric controlled by local image properties; [93] recently presented a generalized version of the Subjective Surfaces technique, while [94] used iterative tangential voting to enhance the protein bound signal followed by evolving fronts.

Fewer techniques deal with automated membrane segmentation in chromogenic IHC, where different and additional challenges arise due to the noise generated by the superposition and diffusion of different stains over the sample and to the presence of unstained portions of the tissues (i.e. where the membranes are negative to the target receptor, or either discontinuously stained). The work presented by [95] is a two-step procedure that first approximates the location of cellular membranes with improved Voronoi, using nuclear boundary as spatial reference, and then refines the segmentation by detecting the nearest stained portions of the membranes by color filtering. Other works use nuclear membranes as reference for approximate localization of the cellular membrane, too, but they rely on simple approximation of membranes' shape: [91] approximate cellular membranes with ellipse, while a work by [96] defines rectangular bounding boxes around the nuclei. The drawback of simple membrane approximation is the lack of robustness with respect to variation of the membranes' shapes, that impacts on the accuracy of membrane protein quantification. Finally, in a recent work by [97] intensity thresholding followed by morphological skeletonization is used to detect the membrane regions in the IHC image, although this is not on a cell by cell basis. The membrane regions are then categorized into two groups: regions with continuous membrane staining and regions with incomplete or weak membrane staining.

Most of the other cell by cell approaches are generally semi-automated in that they need a certain amount of user-intervention to add control points close to the target membrane boundary [98] [99].

8 IHC quantification and semi-quantification

The final step of the IHC workflow is the quantification of the activation of the target antigens (see Fig. 3).

As we anticipated in Section 3, the immunohistochemical test has traditionally been qualitative, consisting in simple observation of the presence and darkness of specific stains within the tissue. However, the rapid evolution of the technique as a valid diagnostic and prognostic tool for tumor marker identification and cancer assessment has rapidly shifted the aim of the IHC test from qualitative to semi-quantitative or quantitative evaluation [10]. Moreover, the availability of computer-aided systems able to extract quantitative information from the images in a fast and reproducible way is contributing to make this shift technically feasible. However, there are still many open issues related to the effectiveness and validity of the obtained information.

The quantitative information extracted from IHC images may be expressed in several ways, including percentages of positively stained cells, intensity of the staining, or either morphological characteristics such as cell size, cell density, and textural features. These features are often used to classify the specimens into a specific number of categories, or scores. Literature provides a plethora of scoring methods, but each laboratory in the standard practice uses its own criteria of evaluation, so that the obtained results are most of the times not comparable at all [100].

A remarkable example for tissue scoring is IHC testing for breast cancer, where the assessment of HER2 (Human Epidermal growth factor Receptor 2) overexpression is widely used to determine the eligibility of the patient for trastuzumab therapy [101]. The American Society of Clinical Oncology and the College of American Pathologists [38] recently published a guideline (later reviewed by the Canadian National Consensus Meeting on HER2/neu testing in breast cancer [39]) containing a recommended algorithm which classifies the specimens tested for HER2 in four classes (0 to 3+) based on both qualitative and semi-quantitative IHC features including uniformity and darkness of membrane staining and percentage of invasive tumor cells.

Several other IHC grading systems have been proposed for the assessment of antibodies related to breast cancer as well as of other pathologies, taking into account features that are generally quantitative or that imply a quantitative or semi-quantitative evaluation of staining intensity, continuity and distribution over the tissue [11]. Among the others, the J-Score [102], the Allred Score [103], the HScore [104], and the scores proposed by [97] and [105].

Although the guidelines are very useful as an attempt of putting some order in the heterogeneity of protocols and clinical trials regarding IHC testing, the way for a standardized quantification appears still long. The judgement about what has to be called "intense" or "weak" intensity, "positive" or "negative" activation, as well as the threshold discriminating between complete and an incomplete staining is generally left to the experience and the subjectivity of the investigator. On the other hand, crisp thresholds are provided in terms of percentages of stained cells, which is not feasible without automated image processing. Consequently, even supposing that all the laboratories follow the guidelines literally, the results obtained by visual evaluation so far are most of the times not comparable [100].

It is widely acknowledged that automated computer-aided techniques have the potential to overcome the subjectivity of visual evaluation, obtaining quantitative features related to the assessment of the antibody directly from the images [11]. The most popular approaches for automated IHC quantification aim at reproducing the decisional process of the pathologist in scoring the tissue slides. These works generally propose two-step procedures including feature extraction techniques, which measure the features of the images that are relevant for the IHC scoring, and a classification step that assigns the score to the sample based on the aforementioned features. The features vary on the base of the type of tissue, generally including rate of positive cells and intensity of staining, as specified by the guidelines for IHC scoring.

The classification is obtained through machine learning techniques, where classifiers are typically implemented and trained on ground truth consisting in scores provided by the pathologists' visual evaluation. The classifier performance is again measured based on agreement with the pathologist's

evaluation, in terms of capability of the classifier to reproduce the visual scoring provided by the human. The proposed approaches are several: [106] classified texture parameters derived from image analysis using a quadratic classifier trained with pathologist's scores. [107] applied gaussian process ordinal regression and supervised neural networks to predict the scores of immunostained tissue microarray spots. [86] and [91] distinguished membrane stained specimens into 1+, 2+ and 3+ scores through Minimum Cluster Distance Classifier with parameters learnt on a pre-scored training dataset. [108] proposed an automated procedure that faithfully replicates the guidelines of the American Society of Clinical Oncology and the College of American Pathologists [38], using SVMs trained with visual scores to distinguish between 2+ and 3+ samples. [40] defined a measure of membrane connectivity and trained a classifier to match automated HER2 scoring with manual scoring performed by five experienced assessors. A recent work by [97] defined its own automated HER2 score based on additive model of membrane connectivity and membrane instensity, and then derived the score cut-offs from a training set of manually scored samples.

Although most of the aforementioned techniques claim a good agreement with the scores provided by the experts, their weakness is the attempt to replicate a decision process that is inherently subjective: in fact, the techniques rely on the pathologists evaluation as ground truth for the scoring, but the scores of the pathologists have been extensively proven to be subjective and affected by extreme inter- and intra-operator variability [100]. As a consequence of that, the proposed gold standard is not robust.

In order to investigate the problem, we run experiments on a pre-scored dataset of seventy H-DAB stained NSCLC (Non-Small-Cell-Lung-Carcinoma) images with cytoplasm activations (see Fig.5 for examples).

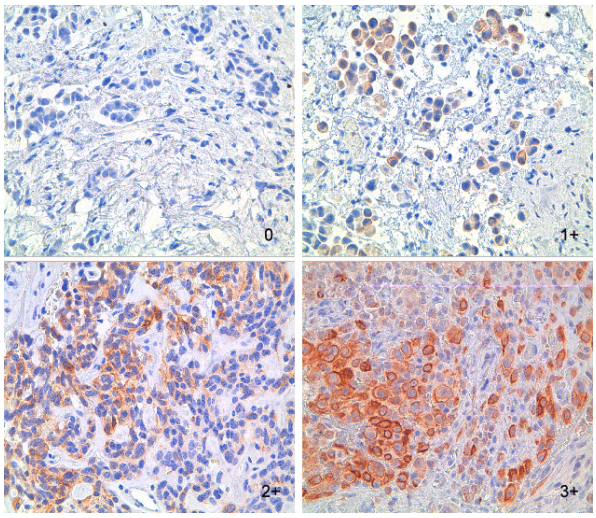

Figure 5: H-DAB stained images of NSCLC samples, with score provided by pathologist.

The scoring features were extracted after tissue and cell segmentation and consisted in rate of positive (i.e. brown stained) cells and average intensity of the brown stain in the positive areas of the sample, in agreement with the guidelines for IHC scoring [38]. The data, grouped by the pathologist' score, are displayed as a collection of points in the scatter-plot of Fig. 6.

As it is shown in Fig. 6, the scatter plot suffers significant overlap of 0 and 1+ HER2 scores, as well as equivocal cases of 2+ scoring (see red and yellow circles, respectively). Even more severe overlaps were experienced by [86] and [91] in images with membrane staining. This definitely confirms the concerns about the the opportunity of using the scores of the pathologist as a golden stardard for the automated quantification techniques.

Several works in literature claim good quantification accuracy based on machine learning meth-

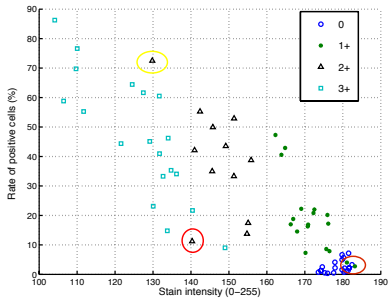

Figure 6: Image features grouped by IHC score (overlaps of different scores in red, ambiguous cases in yellow).

ods that are capable of learning from the visual evaluations of the pathologists [106] [107] [86] [91] [108]. Thanks to the capability of machine learning techniques to capture and replicate the scoring mechanisms of the training data, this approach is generally able to provide a good agreement with the pathologists' evaluations (see Table 1); nevertheless, the results are non-generalizable because the underlying classification model is based on a golden standard, the visual evaluation of the operator, that is inherently not robust.

Table 1: Agreement with pathologist's scoring of some machine learning approaches, in terms of percentage of instances equally classified by the automated technique and the human expert. Agreement was calculated on the NSCLC data shown in Fig. 6 by 10-fold cross-validation.

Classification approach	**agreement with pathologist**
Multilayer perceptron	92.96%
SVM, linear kernel	91.55%
Naive Bayes	90.14%
RBFNetwork	88.73%
fuzzy neural networks	73.30%
SVM, gaussian radial basis function	66.20%

Another major issue related to the automated quantification approaches generally proposed by literature is the significance of the measures of stain intensity. In fact, despite all the guidelines proposing the intensity of the stain as an indicator of the amount of protein activation (in terms of "weak", "medium" or "strong" staining), the translation of this qualitative concept into a quantitative measure extracted from digitalized image is far from being straightforward.

The measurements of staining intensity are generally based on the strong assumption that "n-times darker means m-times more expression"; unfortunately this rather simple relation does not hold in the practice, because the darkness of the stain is determined not only by the Antibody-Antigen reaction per se, but also by a number of other factors that are related to the multiple steps of amplification inherent to immunostaining (see Section 3). As the Antibody-Antigen reactions involve no covalent bonding, it is extremely difficult to isolate and quantify the magnitude of the binding affinity and avidity; moreover temperature, time of incubation, fixation, concentration of Antibodies, batch of Antibodies, crossreactivity and background staining are important factors in determining the relation between antigen activation and immunostaining, therefore they necessarily need to be taken into account in the quantification [11].

This seriously questions the significance of the traditional scoring procedures as proposed by the

guidelines, invalidating the practice of measuring stain intensity from the digitalized images as a direct and absolute indicator of protein expression.

On top of that, the optical characteristics of DAB, the most widely used stain in IHC, are such that there is no linear relationship between the absorbance of the stain when exposed to light and its concentration in the tissue, as expressed by Lambert-Beer law [109]. Densitometrical experiments show that DAB is not a true absorber of light, but rather a scatterer of light [110]; as a consequence, darkly stained DAB has a different spectral characteristics of lightly stained DAB: this implies a highly non-linear behaviour that must be taken into account when interpreting the IHC data.

As a consequence of this, stain-specific calibration charts describing the relation between antibody concentration and stain intensity are necessary for reliable quantitative analysis.

9 Recommendations for reliable automated quantitative IHC

The opinions about the feasibility of a full standardization of IHC is still controversial, because of the many variables that need to be controlled. However, in the last few years there have been extensive efforts towards the solution of the problem. We here try to summarize the main points and provide a few recommendations in this sense.

First of all, a profound re-education of the laboratories and pathologists performing the IHC analysis is needed. In fact, in spite of the remarkable improvements of reagents and of the automation of the sample preparation process, the studies still note extreme inconsistency in the quality of the assays. Moreover, it is pointed out that the main responsibility for such inconsistency rests with the lack of attention to following the most basic directives of the quality assurance programs by a consistent number of laboratories.

Fortunately, the IHC community seems to be now fully aware of the dimension of this problem, and the required actions are being taken. Several consensus conferences have been held in the past few years in order to identify the main causative factors of inconsistency, and to categorize them into pre-analytical, analytical and post-analytical issues [111]. A conference held in late 2006 tried to merge the outcome of the previous meetings with input coming from the laboratories. The result was a series of practical recommendations on how to put the quality standards into practice in the every-day laboratory environment [111].

It is also encouraging that leading providers of antibodies and reagents for IHC are actively participating to the standardization efforts, hosting the consensus conferences and sponsoring international hands-on meetings and practical courses for the pathologists [111].

Provided that the pre-analytical and analytical factors are taken care of, computer-aided analysis is essential to overcome the subjectivity and variability of visual evaluations. As discussed in the previous paragraphs, the technologists have been extensively working on the automation of the image analysis part, but the most common approach is the replication of the interpretation of the human pathologist by means of artificial intelligence or machine learning techniques. As such, the human evaluation (inherently subjective) is used as a golden standard. This is a major limitation: first, because the mechanisms of this scoring process is questionable and not robust; second because this would not fulfil the real potentials of IHC as a valid diagnostic and prognostic tool for tumor marker identification and cancer assessment, able to provide precise and highly localized measures of antigen activations.

As a matter of fact, the only robust way to obtain quantifiable information about stain intensity hinges upon the development of computer-aided techniques that leverage on stain-specific calibration charts. These charts should clearly define the analytical relation between the concentration of the antibody in the tissue and the measured intensity of the stain, taking all the possible effects of the immunostaining amplification steps under control. In absence of this, the only quantification allowed is limited to image features that can be reliably correlated to the pathological process (e.g. morphological features of the positive cells such as nuclear size, cellular size, nuclear-cytoplasm ratio, nuclear density, etc.).

To the best of our knowledge, a full calibration of the IHC process is not achieved yet. Nevertheless,

the research towards the creation of reliable reference standards for IHC is continuous and encouraging. For example, a recent work by [112] experimentally validated the use of peptide controls attached directly to the glass slide as an instrument for practical clinical laboratory quality control. These results suggest that this technology may be valuable in creating fully-standardized controls to quantify IHC analytical variability, including antigen retrieval.

The integration of computer-aided image analysis into a fully-controlled and calibrated immunostaining chain would allow highly accurate quantification of protein expression. This opens much more possibilities to fully-personalized diagnosis and therapy design compared to the current clinical practice, that is still limited to rough discrimination into three to four classes of positivity. Obviously, the fulfilment of this ambitious set of goals requires a more strict collaboration of technologists and image scientists with the pathologists as well as the suppliers of antibody and immunostaining systems; the latters are in fact the ones in charge of implementing the staining calibration process.

On a final note, computer-aided image analysis systems are not meant to replace the expertise of the pathologist. As wisely pointed out by [113], the primary purpose of the automated and semi-automated tools is to complement and support the human expert, that is always expected to provide the final feedback on the performance and trust-wordiness of the automated system. As a consequence of that, the role of the pathologist is far from being diminished by the advent of digital era. As such, it is extremely important that the automated image analysis systems are developed in strict and continuous collaboration with clinical and research pathologists.

10 Conclusions

In the last few years biologists and pathologists are relying more and more on image analysis, and immunohistochemistry (IHC) is nowadays one of the most popular imaging techniques to analyze the presence and activity of target antigens in the tissues, with important applications in the diagnosis and assessment of tumors as well as for several research purposes. However, immunohistochemistry has been traditionally affected by lack of reproducibility due to technological variabilities as well as to the inherent subjectivity of the visual observation; thus the analysis has been limited to qualitative evaluation of the presence and darkness of the target stains within the tissues. The rapid evolution of the technique as a valid diagnostic and prognostic tool has ultimately shifted the aim from qualitative to quantitative; this stressed the demand for the standardization of the overall IHC assay and for the extraction of objective and repeatable measures of protein activity from the IHC images. Computer-aided image analysis has been universally acknowledged for having a fundamental role in solving the IHC standardization issue. In particular, tissue and cell segmentation techniques are precious instruments to identify the regions of interest of the target antigens in the specimens, allowing fully-automated and repeatable measurements at cellular and sub-cellular level. The segmentation approaches vary depending on the specific application and on the morphological and color characteristics of the tissue specimens. In this paper, we provided a critical overview of the techniques proposed by literature.

In theory, provided that the location of interest of the antigens is correctly identified, the problem of quantifying the activation of the antigens by digital image analysis is trivial. Nevertheless, the robustness of immunohistochemical staining raises a number of problems that are generally overlooked in current literature: in fact many of the available techniques for automated IHC scoring are based on weak assumptions. First, in interpreting stain intensity as a direct indicator of protein expression they do not consider the multiple steps of amplification in the immunostaining process. Without a full control of these technological and chemical variabilities, any derived data is misleading. Moreover, the non-linear relation between darkness and real concentration in the tissues of widely used dyes (e.g. DAB) is often unconsidered. Second, they use as a golden standard the scoring of the human experts, that can be subjective and biased by many sources of variability.

In conclusion, computer-aided image analysis has all the potential to lead to a standardized quantitative IHC, but only provided that it is integrated into a fully-controlled and calibrated immunostaining flow. An effective calibration requires the combined efforts of the computer scientists and the providers

of immunostaining systems, and to the best of our knowledge this is not fully available yet. These findings open wide opportunities of further research in the field of quantitative IHC, towards the effective integration of the image processing techniques into the IHC work-flow, and motivate the future efforts of the computer scientists in the field of immunohistochemistry.

11 Conflict of interest statement

None declared.

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