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# Quantification of protein expression in cells and cellular subcompartments on immunohistochemical sections using a computer supported image analysis system

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**Summary.** Quantification of protein expression based on immunohistochemistry (IHC) is an important step for translational research and clinical routine. Several manual ('eyeballing') scoring systems are used in order to semi-quantify protein expression based on chromogenic intensities and distribution patterns. However, manual scoring systems are time-consuming and subject to significant intra- and interobserver variability. The aim of our study was to explore, whether new image analysis software proves to be sufficient as an alternative tool to quantify protein expression. For IHC experiments, one nucleus specific marker (i.e., ERG antibody), one cytoplasmic specific marker (i.e., SLC45A3 antibody), and one marker expressed in both compartments (i.e., TMPRSS2 antibody) were chosen. Stainings were applied on TMAs, containing tumor material of 630 prostate cancer patients. A pathologist visually quantified all IHC stainings in a blinded manner, applying a four-step scoring system. For digital quantification, image analysis software (Tissue Studio v.2.1, Definiens AG, Munich, Germany) was applied to obtain a continuous spectrum of average staining intensity. For each of the three antibodies we found a strong correlation of the manual protein expression score and the score of the image analysis software. Spearman's rank correlation coefficient was 0.94, 0.92, and 0.90 for ERG, SLC45A3, and TMPRSS2, respectively ($p<0.01$). Our data suggest that the image analysis software Tissue Studio is a powerful tool for quantification of protein expression in IHC stainings. Further, since the digital analysis is precise and reproducible, computer supported protein quantification might help to overcome intra- and interobserver variability and increase objectivity of IHC based protein assessment.

**Key words:** Digital image analysis, Automated image analysis protein quantification, Immunhistochemistry

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## Introduction

With the advent of clinically relevant biomarkers the idea of personalized medicine has recently become an area of highest interest (Chin and Gray, 2008; Beroukhim et al., 2010). To make early, rational and correct clinical decisions, a reliable and robust detection of biomarkers is vital. In this respect, the expression profiling of biomarkers by immunohistochemistry has become an integral part of translational research and clinical routine (Golub et al., 1999; Alizadeh et al., 2000; van't Veer et al., 2003).

However, protein quantification by immunohistochemistry remains a semiquantitative approach and is subject to significant intra- and interobserver variability (Becker, 1993). This is mainly due to variations in methodology, the use of manual ('eyeballing') scoring systems, and issues related to pre-ß and post analytic aspects of this technique (Hammond et al., 2010). In response to this, recent developments in image acquisition technology (Yagi and Gilbertson, 2005; Taylor and Levenson, 2006; Walker, 2006), data management (Kayser et al., 2008; Rojo et al., 2008; Wienert et al., 2009) and computed image analysis (Mulrane et al., 2008; Baatz et al., 2009) have lead to an increasing interest in digital pathology. Since the first

efforts of computed protein quantification in 1987 (Franklin et al., 1987), several studies comparing visual and computer supported image analysis have been published, mainly focussing on the protein expression of estrogen receptor (McClelland et al., 1990; Kohlberger et al., 1999; Mofidi et al., 2003; Diaz et al., 2004; Turbin et al., 2008) and HER2/neu in breast cancer (Wang et al., 2001).

In this study, we are the first to explore whether the digital image analysis software Tissue Studio v.2.1 (Definiens AG, Munich, Germany) proves sufficient as an alternative tool to quantify protein expression in cells and cellular subcompartments on immunohistochemical stained sections. In detail, immunostainings of ERG (Furusato et al., 2010; Park et al., 2010), which expresses in the nucleus, SLC45A3 (Kalos et al., 2004), which expresses in the cytoplasm and TMPRSS2, which expresses in both cellular subcompartments, were independently quantified by both, the image analysis system and a pathologist on the same cohort of 614 prostate cancer specimens. Subsequently, we compared the results of visual and computer supported scoring.

## Materials and methods

### *Cohort*

We assessed a cohort of 630 patients diagnosed with prostate cancer for protein expression of ERG, SLC45A3 and TMPRSS2. The cohort was comprised of tumor material from Middle European patients who underwent radical prostatectomy between 1999 and 2005 at the Charité Hospital in Berlin. The detailed demographics of this cohort were described recently (Kristiansen et al., 2008).

### *Tissue microarray (TMA) construction*

After surgical removal, radical prostatectomy specimens were immediately forwarded to routine pathology. Specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. After routine pathology diagnostics, the formalin-fixed paraffin-embedded specimens were stored at room temperature in the archive under the same conditions until their retrieval. Subsequently, formalin-fixed paraffin-embedded prostate cancer specimens were cut into 3 $\mu$m thick sections, mounted on slides, and stained with hematoxylin and eosin. Prior to TMA construction, prostate cancer foci as well as benign regions were marked by an experienced pathologist on the corresponding haematoxylin and eosin stained slides. Tissue core biopsies, each 1.8 mm in diameter, were taken from the corresponding donor block and placed into a tissue microarray recipient block using a semiautomatic tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA). In detail, to avoid loss of case numbers due to insufficient staining reactions or other sources of error, each patient was represented with at least six tissue cores (two benign, two high grade prostatic intraepithelial neoplasia (HGPIN), and two invasive prostate cancer tissue cores). Only cases with at least one assessable invasive prostate cancer tissue core were included in the study. Benign and HGPIN tissue cores served as control.

### *Immunohistochemistry (IHC)*

For IHC experiments, 3$\mu$m thick tissue sections of the TMAs were placed onto superfrost slides. IHC was conducted with the Ventana-Benchmark automated staining system (Ventana Medical Systems, Tucson, AZ, USA). The following primary antibodies were used: rabbit anti-ERG (Abcam Limited, Cambridge, UK; dilution 1:100), rabbit anti-TMPRSS2 (Abcam Limited, Cambridge, UK; dilution 1:4000) and mouse anti-human SLC45A3 (DAKO A/S, Glostrup, Denmark; dilution 1:100). Slides were counterstained with haematoxylin (Ventana Medical Systems, Tucson, AZ, USA) and the proper alkalinity was ensured by washing with Bluing reagent, pH=8.0. An additional dehydration step included initial ethanol wash cycle (70%, 80%, 96% and 100% Ethanol), followed by 4 xylene wash steps. The secondary washing was performed using CC1 buffer (Cell conditions 1) and TBE buffer with pH= 8.4 (Ventana Medical Systems, Tucson, AZ, USA). Primary antibody detection was performed using the iView™ DAB detection kit (Ventana Medical Systems, Tucson, AZ, USA). To avoid different experimental conditions, all TMA experiments were performed simultaneously on the same staining device (Ventana) in one run. For ERG IHC, nuclear immunoreactivity of endothelial cells located in most TMA tissue cores served as an intrinsic positive control. Likewise, for TMPRSS2 and SLC45A3 IHC, benign prostatic glands expressing the protein served as positive controls. Criteria for sufficient staining were antibody binding specificity, tissue morphology and overall staining quality.

### *Visual and computer supported evaluation of the immunohistochemical staining*

After performing immunohistochemistry on the sections, the slides were digitalized at 20x magnification using the Zeiss MIRAX DESK scanner (Carl Zeiss, Oberkochen, Germany). The protein expression of ERG, SLC45A3 and TMPRSS2 was then assessed in a two step analysis:

First, all slides were evaluated visually by an experienced genito-urinary pathologist (G.K.) with a standard bright-field microscope. A semiquantitative, four-stage scoring system was applied, ranging from negative immunoreactivity (0) to strong immunoreactivity (3+) of the tumor cells or cellular compartments (i.e., tumor nuclei for ERG, tumor cell cytoplasm for SLC45A3 and both compartments for TMPRSS2).

Second, semi-quantitative image analysis software

(Tissue Studio v.2.1, Definiens AG, Munich, Germany) was independently applied to all corresponding digitalized slides, obtaining a continuous spectrum of average brown staining intensity in arbitrary units (maximum range of readout 0.000-3.000). This software allows to specifically mine for staining intensities in different compartments of the cell, i.e. cell nuclei, cytoplasm, membranes, or whole cells in a user specified region of interest (ROI). In order to exclude most stromal cells and benign areas from the evaluation, an experienced genito-urinary pathologist (S.P.) targeted ROIs for each TMA core prior to the analysis. Further, staining thresholds (hematoxylin, DAB density) and morphological criteria (elliptic shape, area) were introduced in order to exclude the vast majority of benign cells included in the ROI. For statistical evaluation, we only selected TMA cores where at least 50 cells passed the filtering process. If differences were observed between tissue cores of the same patient with regard to the staining pattern, the arithmetic mean was calculated for the respective duplet. Despite the slide scanning, all steps of the digital analysis, including the manual region of interest (ROI) selection, were performed on the same computer (Windows 7 based environment, 24" monitor, resolution 1920x1080).

### *Statistics*

In order to assess the results of the visual and computer supported analysis for a possible correlation, we used the Spearman's correlation test (SPSS Inc., Chicago, IL).

## Results

ERG, SLC45A3, and TMPRSS2 were expressed in 332/630 (52.3%), 624/628 (99.3%), and 621/624 (99.5%) cases, respectively. Based on the visual assessment as performed by the pathologist, the mean protein expression of ERG, SLC45A3 and TMPRSS2

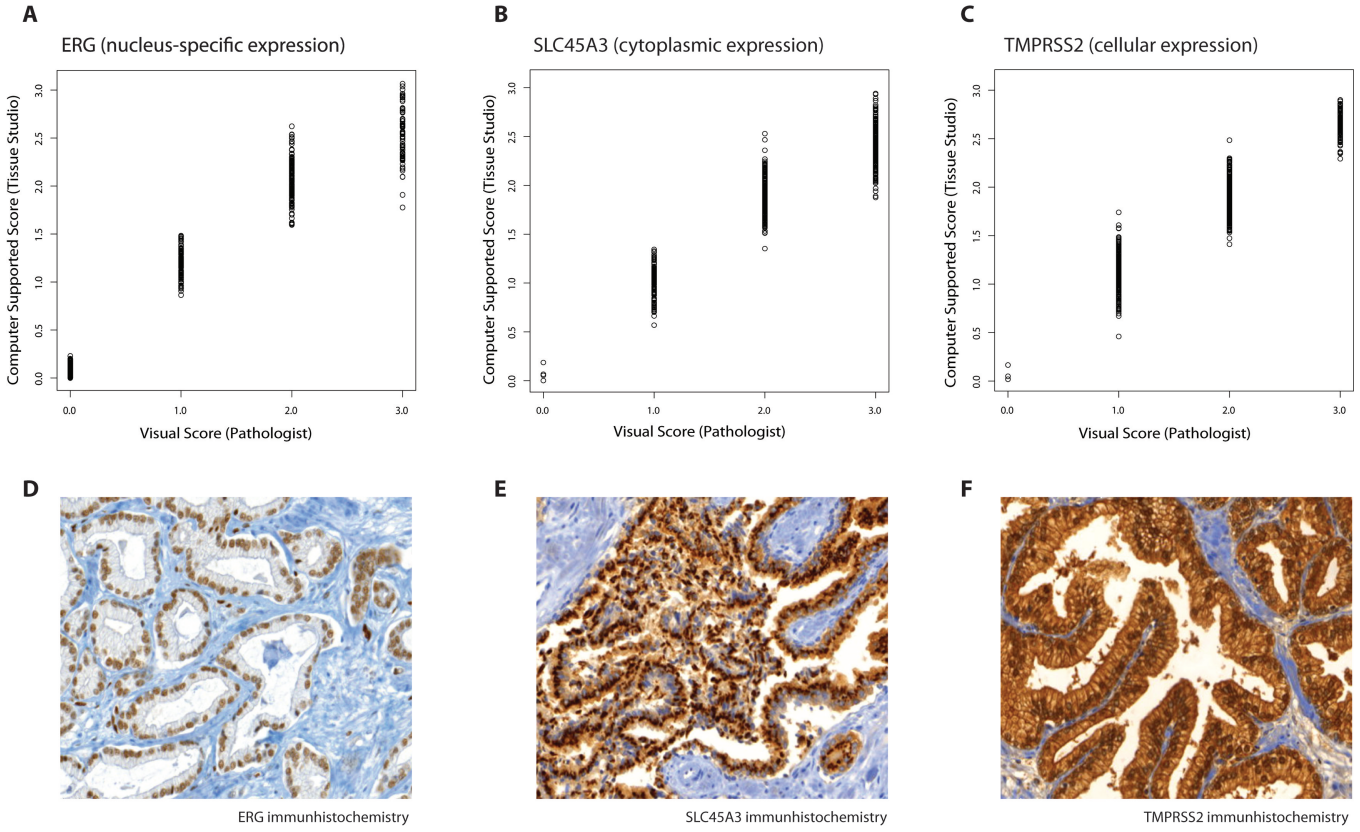


**Fig. 1.** Comparison of computer supported and visual quantification of protein expression on immunhistochemically stained sections. **A-C.** Scatter plots illustrating the computed scores (continuous spectrum, maximum range of readout 0-3) as acquired by the image analysis software Definiens Tissue Studio and the visual scores (four-stage system, 0-3) as aquired by a pathologist. Regardless of whether assessing immunostainings with antibodies that are specific to the nucleus **(A),** cytoplasm **(B)**, or expressed in both cellular compartments **(C)**, computed and visual scoring results correlated significantly (Spearman's rank correlation coefficient 0.94, 0.92, and 0.90, respectively). **D-F.** Representative tissue sections (20x magnification) of prostate cancer specimens immunohistochemically stained with the nucleus specific anti-ERG antibody (visual score: 2), the cytoplasm specific anti-SLC45A3 antibody (visual score: 3), and the anti-TMPRSS2 antibody (visual score: 3).

was 1.08, 2.30 and 1.82 respectively. Based on the computer supported assessment via Tissue Studio, the mean protein expression of ERG, SLC45A3 and TMPRSS2 was 0.93, 2.12, and 1.64, respectively.

For each of the three antibodies, we found a strong correlation of the visual protein expression score and the score of the computer supported analysis. Spearman's rank correlation coefficient was 0.94, 0.92, and 0.90 for ERG, SLC45A3 and TMPRSS2, respectively ($p<0.01$) (Fig. 1).

In terms of time that it took to assess the specimens with both approaches, digital processing (including scanning, manual ROI selection, digital processing, and data extraction) took approximately 3-4 hours per TMA. However, an experienced pathologist is only needed for manual ROI selection, which approximately takes 1-2 hours. When manually assessing the same TMA on a microscope, the observer needed 1-2 hours.

## Discussion

The first efforts to digitally quantify protein expression were undertaken over two decades ago (Franklin et al., 1987; McClelland et al., 1990). However, until more recently, the lack of sufficient technologies and validated standards in digital image acquisition and analysis did not allow the reliable inclusion of digital image assessment in research and routine diagnostics (Franklin et al., 1987; Yagi and Gilbertson, 2005). With the rise of advanced digital scanners (Yagi and Gilbertson, 2005; Taylor and Levenson, 2006; Walker, 2006), image management tools (Kayser et al., 2008, Rojo et al., 2008, Wienert et al., 2009) and image analysis software (Mulrane et al., 2008; Baatz et al., 2009), the importance of digital pathology is substantially growing. While visually immunohistochemistry based protein quantification remains a rather subjective approach that is characterized by significant intra- and interobserver variability (Becker, 1993), digital image assessment has a huge potential to overcome these limitations. Nevertheless, especially with respect to the increasing number of clinical decisions that are based on protein expression analysis (Golub et al., 1999; Alizadeh et al., 2000; van't Veer et al., 2003), a comprehensive validation of digital imaging techniques and software is mandatory before their everyday application in clinical routine.

Here, we are the first to assess whether the image analysis software Tissue Studio v.2.1 is an appropriate alternative or addition to visual protein quantification of cells and cellular subcompartments. In detail, we compared the immunoreactivity of 614 prostate cancer specimens as scored by a pathologist with the results of the digital analysis. We found a strongly significant correlation between visual and computer supported protein quantification, whether quantifying the protein expression specific to the nucleus, cytoplasm or both compartments of the cell (Fig. 1). Previous studies applying other image analysis platforms, mainly focusing on estrogen receptor and HER2/neu protein expression, have reported similar results (McClelland et al., 1990; Kohlberger et al., 1999; Wang et al., 2001; Mofidi et al., 2003; Diaz et al., 2004; Turbin et al., 2008).

Notably, the best correlation between the visual and computer supported results was achieved when a pathologist manually selected the region of interest (ROI) prior to the digital image analysis. While automatic ROI detection is an extra in computer supported assessment, we experienced inaccuracy and inconsistency in significant subsets of specimens (e.g., mix-up of malignant and benign lesions) which considerably limits observer-independent analysis. In particular, the quantification of proteins that are not restricted to malignant tissue (e.g., SLC45A3 and TMPRSS2) remains one of the biggest challenges in this regard. This is mainly due to the fact that in such a scenario the image analysis system is required to specifically distinguish cells based on marker-independent criteria, such as cell morphology and tumor architecture. Conversely, automatic ROI worked reliably when used for the quantification of proteins that are tumor-specific (e.g. ERG). Notably, with the scanning and processing of the specimens, digital analysis leads to an overall increase in time than it would take to assess specimens 'eyeballing' based. However, the time needed, of course, strongly depends on the size of the TMA, the performance specifications of the computer environment, as well as the morphologic and software experience of the user. Notably, a pathologist is only needed for the manual ROI selection, while most of the other time-intensive steps could, for example, be performed by a technician. Nevertheless, even though manual ROI selection might be time demanding and requires the presence of a pathologist, it enhances reproducibility of results and helps to increase objectivity and accuracy of the assessment as compared to non-computer supported assessment.

Furthermore, due to the continuous spectrum of the results, computer supported analysis allows a post-analytical setting of scoring categories (theoretically, 1000+ categories are possible) and thresholds, while the visually assessing pathologist is mostly restricted to a pre-defined scoring system (e.g., 0,1,2,3+). As previously shown by us, these advantages make data interpretation and statistical evaluation much more flexible and enable multiparametric investigation as well as the enhanced identification of underlying correlation (Maier et al., 2011; Wilbertz et al., 2011). In this respect, we suggest that digital analysis might be able to assess individual and potentially clinically relevant differences between patients that might have been overseen by manual analysis.

Besides the above mentioned issues that might limit the reliability of computer supported results, the pathologist has to keep in mind that changes in pre-analytical sample processing do not only affect

conventional (visual) scoring but also have a significant impact on digital staining readouts. In our experience, a computer supported analysis is more detailed than a visual assessment, but demands a well standardized environment. In particular, working in a tissue microarray based setting, all sections should preferably be of the same thickness (3-6 $\mu$m), stained using the same protocol with the same device and at the same time, digitally scanned using a similar device, and contain specimens that are derived from the same institution (e.g., due to a similar fixative conditions). However, even in tissue microarray based experiments, variations in staining intensities cannot be fully avoided, as immunoreactivity is enzymatic based and influenced by a number of factors (such as minor local variances in fixation, age, incubation times, and quality of specimens, etc.). In this respect, the software itself cannot estimate whether the variation within or between different tissue spots of the same patient is appropriate or not. This step still requires the presence of an experienced pathologist. In the present study, if differences were observed between tissue cores of the same patient with regard to the staining pattern, the arithmetic mean was calculated for the respective duplet.

Notably, the software also works in a fluorescent environment, i.e. FISH or Immunofluorescence slides can be digitalized and processed, both in a TMA or whole slide based setting. In this respect, various analyses, such as the quantification of gene amplification assays or immunofluorescent reactivities are possible (data not shown). As to the imaging of protein movement inside a given cellular subcompartment (e.g., imaging of receptor internalization or compartment analysis of temporal activitiy), the capabilities of the software are rather restrictive. While it is for instance possible to relate the immunoreactive area of the cytoplasm in comparison to the nonreactive cytoplasm area, it is not possible to automatically extract the exact location of immunoreactivity within a given compartment. However, the software goes along with a Developer Tool that allows to manually enhance the software with a multitude of scripts and imaging functions. These settings are highly experimental and demand an advanced level of programming skills and image analysis experience. In this study, we limited our experiments to the pre-manufactured version of the software, which is user-friendly and still supports a great variety of imaging opportunities.

In synopsis, the present study shows that the digital image analysis platform Tissue Studio is a powerful alternative for the quantification of protein expression in immunohistochemically stained sections. Further, since the digital image analysis is reproducible, computer supported protein quantification might help to overcome intra- and interobserver variability and increase the objectivity of immunohistochemistry based protein assessment. Future advancements in image acquisition and digital processing are warranted, particularly with regard to an automatic ROI detection, but beyond doubt have the potential to revolutionize possibilities in routine histopathology and translational research.

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