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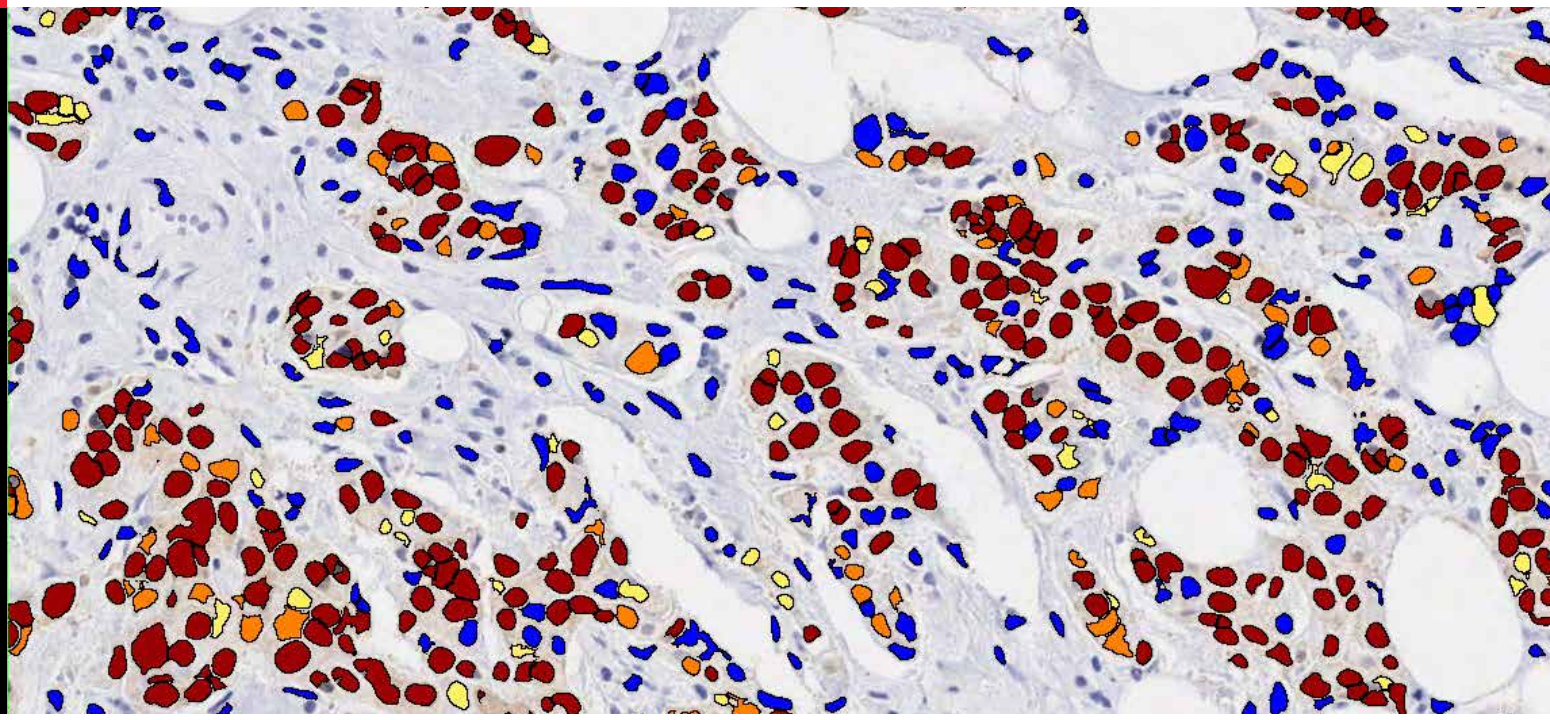

Use of Aperio Image Analysis in Peer-Reviewed Breast Cancer Research

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Introduction

Worldwide, breast cancer is one of the most common cancer diagnoses among women[1]. Breast cancer is also one of the most studied cancers. A review of annual research spending in the United States of America shows more funding allocated for breast cancer research than for any other cancer type[2]. The drop in breast cancer mortality in the United States by 34% between 1990 and 2010[3] has been attributed to improvements in both detection and treatment, which reflects the high volume of research. However breast cancer remains a leading cause of cancer death in women[1,3], highlighting the need for continued research and use of new technologies to drive treatment breakthroughs.

Researchers across a variety of fields are increasingly using quantitative image analysis tools to support their research. These tools allow researchers to measure biomarker data in a quantitative manner, which offers a number of advantages over manual qualitative or semi-quantitative review. This includes generation of research data that are standardized and reproducible, as well as reduction of inter- and intra-observer variability and subjectivity. In addition, it offers the ability to analyze histology images in a high-throughput fashion with minimal user interaction, reducing manual effort and analysis turnaround time. With the emergence of digital pathology, users now have access to a wide assortment of computer-assisted image analysis options, from basic pixel counting to highly specialized tools for specific applications.

Leica Biosystems Aperio Digital Pathology offers a suite of customizable algorithms, which can be trained by the user to work across a range of tissue and biomarker types. The flexibility of these algorithms makes them ideal for research applications, allowing scientists to utilize each tool for multiple studies.

This review paper addresses recent peer-reviewed publications on use of Aperio Image Analysis algorithms for breast cancer research applications, such as elucidation of tumorigenic pathways, identification of novel prognostic indicators, and development of therapeutic targets.

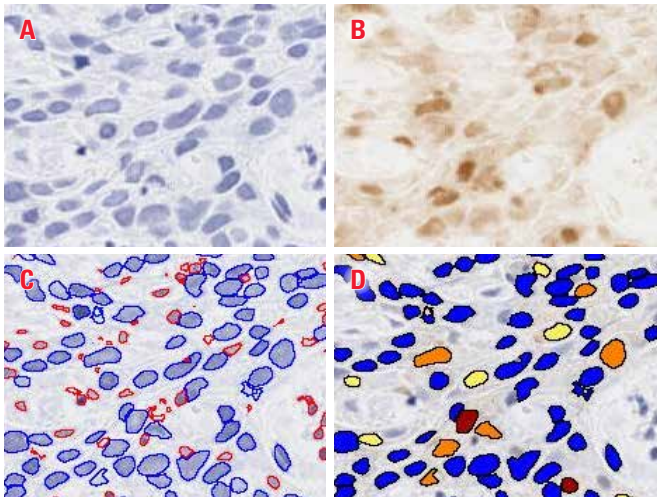

Figure 1: *Tuning the Aperio Nuclear Algorithm to detect nuclei in tissue sample. (A) Detection of nuclear counterstain (Hematoxylin). User is able to tune algorithm to other counterstains. (B) Detection of positive nuclear stain (DAB). User is able to tune to detect other chromogens. (C) Automatic detection of nuclei by the algorithm; the user can optimize the detected nuclei to exclude (red) based on size and shape. (D) Final mark-up of nuclei to include classification as strong (red), moderate (orange), weak (yellow), or negative (blue) staining, based on user-selected intensity cut-offs.*

Mechanisms of Pathogenesis

Digital pathology tools are employed by researchers to examine phenotypic aspects of breast cancer formation in *in vivo* animal models, including development of primary breast cancer and tumor metastasis. Lyons *et al.*[4] examined the role of Cyclooxygenase-2 (COX-2) in development of an invasive phenotype of Ductal Carcinoma In Situ (DCIS). They used the Aperio Color Deconvolution Algorithm to analyze intensity of COX-2 staining as well as a number of other tumor progression markers, and results supported the hypothesis that the COX-2 pathway promotes cancer development. The same group performed a study to examine COX-2's specific role in DCIS lymphatic metastasis[5], using the same image analysis methodology.

Cellular proliferation and apoptotic cell death pathways are extensively studied in breast cancer research to understand the etiology of tumor growth and progression. The role of proliferation marker Ki67 has been a key aspect studied, often in recent years with the aid of Aperio Image Analysis. Dominatskaya *et al.*[6] and Northey *et al.*[7] both utilized the Aperio Nuclear Algorithm to evaluate Ki67 expression. Northey *et al.*[7] additionally used the

same algorithm to measure TUNEL positive nuclei, capitalizing on the ability of the algorithm to be tuned for different biomarkers. Other studies have utilized the Aperio Positive Pixel Algorithm to quantify expression of a wide range of biomarkers in pathogenesis, including CD151, Cleaved Caspase-3 and CD31[7,8].

A number of studies have also used image analysis to investigate effects of tumor microenvironment on breast cancer progression, such as Maller *et al.*[9], who used the Aperio Color Deconvolution Algorithm to evaluate increases in junctional E-Cadherin, a known marker of tumor suppression, in breast tissue collagen during pregnancy. Sun *et al.*[10] used the Aperio GENIE histology pattern recognition tool to measure composition of normal breast tissue adjacent to tumorous tissue in 118 patients, separating epithelium, adipose tissue, non-fatty stroma and glass. After first training Aperio GENIE on a subset of slides, they ran the algorithm on the full digital image set, and compared these results with a pathologist read of both the glass slides and digital slide images, to assess performance. The results showed strong correlation between all three methods, and the authors chose to use digital image analysis for the full study, noting that "*compared with digital assessment, visual assessment by human eye on regular H&E slides of small percentages is weaker*".

Pang *et al.*[11] used several Aperio algorithms to quantify various breast tissue attributes in women at high risk of breast cancer. The Aperio Positive Pixel Algorithm was employed to analyze tissue composition on H&E slides, with strongly stained pixels classified as epithelium, moderate or weakly stained pixels as stroma, and negatively stained pixels as fat. The authors were able to calculate the proportion of each tissue type from the total number of pixels in the section. The Aperio Microvessel Analysis Algorithm was used to measure angiogenesis, with vascular tissue stained by CD31, while the Aperio Nuclear Algorithm analyzed a number of immunohistochemical markers, including ER-alpha, ER-beta, PR, and Ki67. In this study, image analysis was used to quantify a variety of different attributes, contributing to a detailed view of the tissue phenotype.

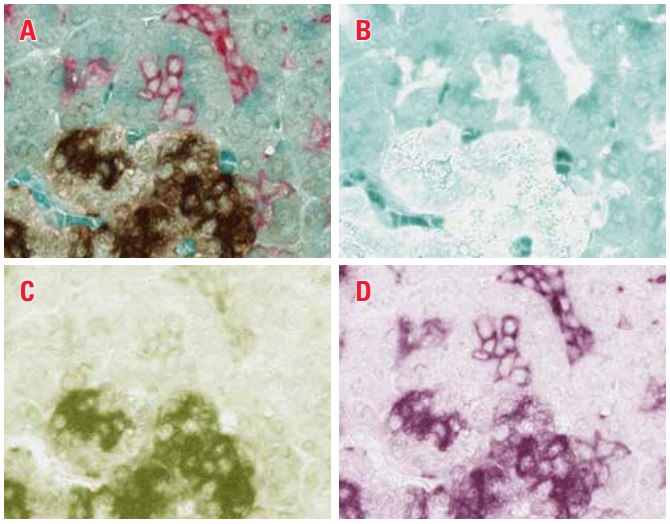

Figure 2: *Separation of trichrome staining using the Aperio Color Deconvolution Algorithm. (A) Original triple-stained tissue. (B-D) Separated (deconvolved) stains: (B) location of Crystal Light Green stain, (C) location of DAB stain and (D) location of Fast Red. As well as visual separation, the Color Deconvolution Algorithm will provide information on area and intensity of staining for each individual stain.*

Prognostic Indicators

Research into identification of potential new markers that could act as indicators of outcome for patients in the future is a vital step towards development of personalized treatments. Automated image analysis tools can be used to quantitatively measure these biomarkers under research investigation.

Brennan *et al.*[12] examined the protein survivin as a marker of improved prognosis in breast cancer. The role of survivin as a prognostic indicator had long been considered controversial. The authors proposed that the differential expression of the marker across nucleus and cytoplasm could be used as an indicator of outcome, and aimed to measure staining of each compartment quantitatively using image analysis. They used the Aperio Positive Pixel Algorithm to quantify staining in subcellular compartments, and found that the ratio of cytoplasmic to nuclear staining was correlated with other markers of outcome. The group then performed a larger study on a set of 512 patients diagnosed with primary invasive breast cancer[13], to further support their initial findings. The authors noted that manual analysis of the cytoplasmic to nuclear ratio is challenging, and that "*the introduction of digital imaging devices and computer-assisted image analysis has provided a major advance towards quantitative description of IHC signals*". In this study they used Aperio GENIE to differentiate tumor and stromal regions in tissue, allowing them to accurately and automatically identify the correct cohort of cells,

followed by the Aperio Positive Pixel Algorithm to measure expression of survivin within only the cells of interest, while accounting for the differentiated subcellular localization of the protein.

Numerous other studies have used Aperio Image Analysis tools in evaluation of potential prognostic markers, using populations of breast cancer patients. O'Leary *et al.*[14] analyzed tumor tissue microarrays (TMAs) containing samples from 442 patients using the Aperio Color Deconvolution Algorithm to quantify areas of weak, moderate and strong staining of Peroxiredoxin-1 (PRDX1), a potential indicator of improved survival. With this quantitative data, they were able to correlate increased PRDX1 expression with improved relapse-free survival in ER-positive tumors.

Putluri *et al.*[15] examined a cohort of 192 samples for expression of Ribonucleotide Reductase Subunit M2 protein (RRM2), finding a correlation with tamoxifen-resistance in tumors. Using the Aperio Cytoplasmic Algorithm they quantified intensity of RRM2 staining, specifically within the cytoplasmic compartment of the tumor cells. Román-Pérez *et al.*[16], used 72 patient samples to perform a study of gene expression in tumor microenvironment, as a potential indicator of outcome. They used the Aperio Nuclear Algorithm to measure expression of Twist-related protein 1 (TWIST1) in epithelium and stroma, indicating that a specific microenvironment subtype was associated with higher tumor proliferation.

Image analysis tools can also be used to in conjunction with manual assessment of prognostic biomarkers, as demonstrated in a study by Lanigan *et al.*[17]. They assessed the potential value of the protein

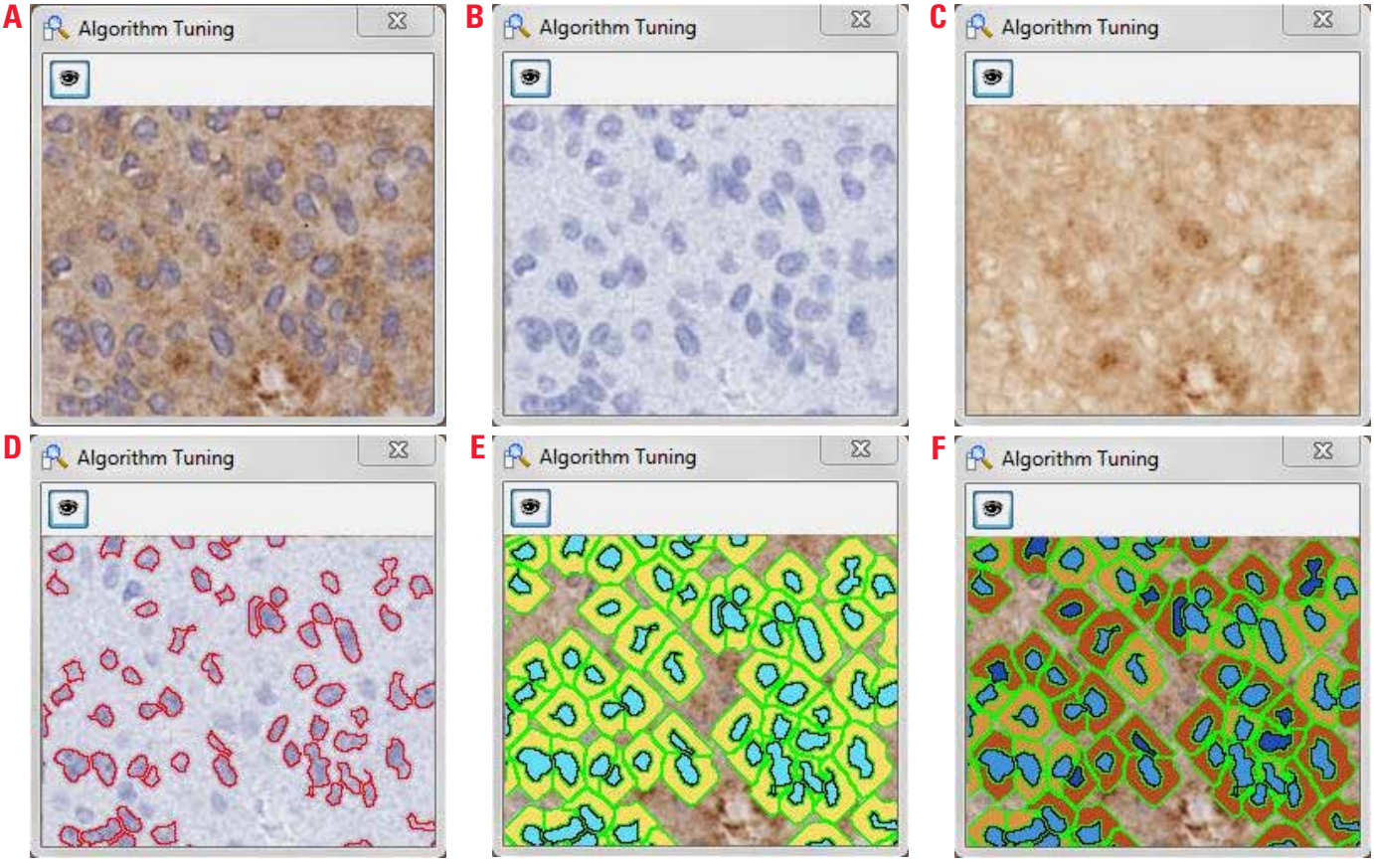

Figure 3: *Tuning the Aperio Cytoplasmic Algorithm. (A) Original tissue stained with DAB and Hematoxylin counterstain. (B) Detection of nuclear counterstain (Hematoxylin). The user can tune the algorithm to detect other counterstains. (C) Detection of positive stain (DAB). The user can tune to detect other chromogens. (D) Automatic detection of nuclei by the algorithm. The user can optimize the detected nuclei to suit their counterstain and tissue. (E) User-defined cytoplasmic separation, i.e. the distance from the nucleus to be classified as cytoplasm. (F) Final mark-up of modeled cells showing in-depth classification of cellular cytoplasmic staining; the intensity of the cytoplasm is denoted by a variation from yellow to red, and the nuclear staining is illustrated by light to dark blue.*

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Muscle Segment Homeobox 2 (Msx2) as an indicator of clinical outcome, using two independent observers to assess nuclear and cytoplasmic Msx2 expression, and then analyzed the images with the Aperio Colocalization Algorithm "*to control for the subjectivity inherent in the manual scoring process*". They found a strong correlation between manual and automated analysis, and results indicated that increased cytoplasmic expression of Msx2 was associated with improved outcome. Cawthorn *et al.*[18] examined two distinct markers, Decorin and Endoplasmin, for their prognostic value, with manual read by two pathologists on a semi-quantitative scale, and supporting quantitative data produced using the Aperio Positive Pixel Algorithm. In these studies, use of image analysis acted as a control for manual review, providing the researchers with objective biomarker quantitation and greater confidence in their results.

Therapeutic Targets & Response

Digital image analysis tools also play an important role in identification of novel therapeutic targets for breast cancer. Examination of novel treatment targets, including evaluation of tumor response to proposed therapies, requires that image analysis algorithms be flexible and customizable to a range of biomarkers.

Kalra *et al.*[19] were able to tune the Aperio Positive Pixel Algorithm to quantify a number of IHC markers, including pAKT Serine 473, BAD, Cleaved Caspase-3, TWIST, and p(ser9/21)GSK3-alpha-beta serine 9/21, to uncover the mechanism of action of Integrin Linked Kinase (ILK) inhibition by the molecule QLT0267. The authors noted that the large number of signaling pathways and downstream molecules affected by ILK presented a challenge in identifying the specific mechanism of tumor suppression by ILK inhibition, and a secondary aspect of their study was to establish a number of tools, including digital image analysis, that could be combined to evaluate multiple endpoints in a drug study. They commented that "*visual scoring proved time-consuming and was less effective at picking up the subtle changes in marker expression, whereas digital quantification enabled the use of high-resolution, high-magnification images to count positive pixels and to assess for changes in marker localization*". This study demonstrated the value of a flexible, tunable analysis algorithm to evaluate multiple markers in a quantitative and high-throughput manner.

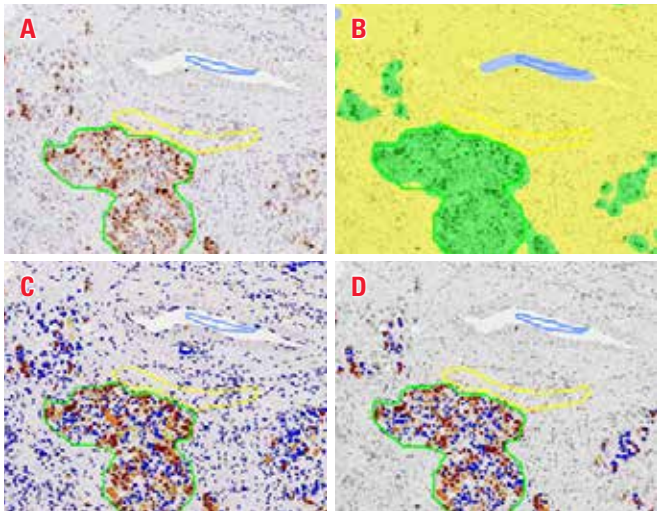

Figure 4: *Use of Aperio GENIE in conjunction with the Aperio Nuclear Algorithm to quantify biomarkers in tumor. Image A shows the original tissue sample with regions of different tissue type and glass regions annotated for Aperio GENIE training. Image B shows the tissue after Aperio GENIE training, with tumor (green), stroma (yellow), and glass (blue) identified. Image C shows analysis of the region with the Aperio Nuclear Algorithm, showing identification of nuclei within both tumor and non-tumor (stroma). Image D illustrates the effect of pre-processing with Aperio GENIE prior to Nuclear Algorithm analysis. Aperio GENIE automatically identifies the tissue of interest (in this case, tumor) and the Nuclear Algorithm is run only within these regions.*

Other studies have utilized the Aperio Nuclear Algorithm to quantify multiple markers in studies of potential therapeutic targets. Examples include Hahm *et al.*[20], who examined expression of TUNEL and Proliferating Cell Nuclear Antigen following Withaferin A treatment, and Cochrane *et al.*[21], who used the algorithm in a study of Androgen Receptor (AR) inhibition by the compound Enzlutamide, to quantify both AR and Ki67, as well as quantifying Cleaved Caspase-3 with the Aperio Positive Pixel Algorithm.

The Aperio GENIE tool for histology pattern recognition has also been used in studies of novel therapies, particularly in combination with other algorithms. Lloyd *et al.*[22] examined tumoral bloodflow as a potential therapy target for ER-positive tumors, using Aperio GENIE to identify tumor tissue and the Aperio Microvessel Analysis Algorithm to measure angiogenesis. Similarly, Mignion *et al.*[23] measured tumor chemotherapeutic response, using Aperio GENIE to classify regions of tumor, necrosis and non-target tissues,

and the Aperio Positive Pixel Algorithm to quantify both Cleaved Caspase-3 and percentage of necrotic area in the whole tissue section. In these studies, use of Aperio GENIE as a pre-processing tool allowed researchers to more accurately target regions of interest for IHC analysis.

Comparison of Image Analysis with Manual Review

Image analysis tools are capable of accurately quantifying biomarker staining in tissue, with numerous studies demonstrating strong correlation between manual review and automated analysis.

Nassar *et al.*[24] performed a multisite study of 260 breast tissue specimens, comparing quantification of ER and PR by Aperio Image Analysis with blinded read by three pathologists, and found substantial correlation between the automated and manual methods. The same group[25] evaluated HER2 automated analysis in their 260 specimen cohort, finding that not only were automated analysis results substantially equivalent to manual read, but that the availability of quantitative data improved inter-pathologist agreement.

Fasanella *et al.*[26] used the Aperio Nuclear Algorithm to quantify Ki67 expression in 315 breast cancer samples, which had previously been evaluated by a pathologist, and reported a high level of correlation between manual and automated analysis.

Lloyd *et al.*[27] compared ER and HER2 scoring by digital image analysis with both manual read by two pathologists and gold standard HER2 FISH, using Aperio GENIE to identify tumor areas and the Aperio Nuclear and Membrane Algorithms to quantify the biomarkers of interest within the tumor. They found that all of the image analysis results fell within acceptable range of pathologist manual read.

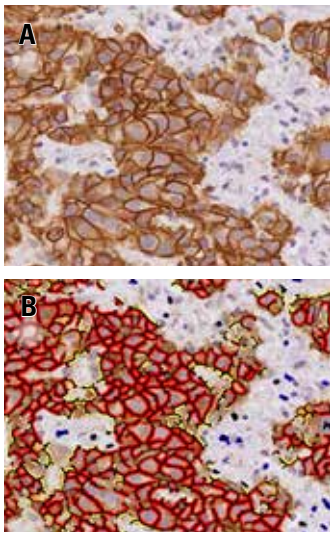

Figure 5: *Visualization of analysis by the Aperio Membrane Algorithm. (A) Original tissue with DAB-stained membrane and hematoxylin counterstain. (B) Final mark-up of analyzed tissue. The algorithm automatically detects cell membrane and based on tuning by the user, it classifies membrane areas as strong (red), moderate (orange), or weak (yellow) staining. Outputs include cell counts and % completeness of membrane staining.*

Laurinavicius *et al.*[28] used similar methodology in their image analysis, employing Aperio GENIE in combination with the Aperio Nuclear and Membrane Algorithms to quantify a panel of breast biomarkers, including HER2, ER, PR and Ki67. This study looked at potential development of a multi-marker expression profile for Ductal Carcinoma, and examined the potential of image analysis to provide more accurate and quantitative results. Their conclusions described image analysis as "*an efficient exploratory tool clarifying complex interdependencies in the breast cancer carcinoma IHC profiles*".

Conclusion

Digital image analysis tools are increasingly being recognized for their utility in various research fields. The ability to quantify biomarker expression and produce detailed, reproducible data makes these tools invaluable to cancer researchers.

This review outlines the extensive usage of Aperio Image Analysis tools across the field of breast cancer research. The algorithms can be flexibly configured for a wide variety of highly specific use cases including novel biomarkers, animal and human models, tissue microarray and whole tissue research. Aperio tools are the most widely referenced in peer reviewed publications worldwide. Over 400 publications reference Aperio Image Analysis Algorithms, demonstrating their flexibility and reliability for quantitative and semi-quantitative research image analysis.

Statements regarding the comparative utility or performance of LBS products are the opinions of the respective study authors based on experience during the study and are not intended as performance claims by Leica Biosystems.

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