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Use of Aperio Image Analysis in Peer Reviewed Breast Cancer Research

INTRODUCTION

Worldwide, breast cancer is one of the most common cancer diagnoses among women[1]. Breast cancer also is 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 level of cancer 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 breast cancer breakthroughs.

Researchers across a variety of fields, are increasingly using quantitative image analysis tools to support their research methods. These tools allow researchers to measure biomarker data in a truly objective, quantitative fashion, which offers a number of advantages over manual qualitative or semi-quantitative biomarker review. This includes generation of research data that are highly standardized and reproducible, 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 study 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 ePathology 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, including applications such as elucidation of tumorigenic pathways, identification of novel prognostic indicators, development of therapeutic targets, and validation for clinical decision support.

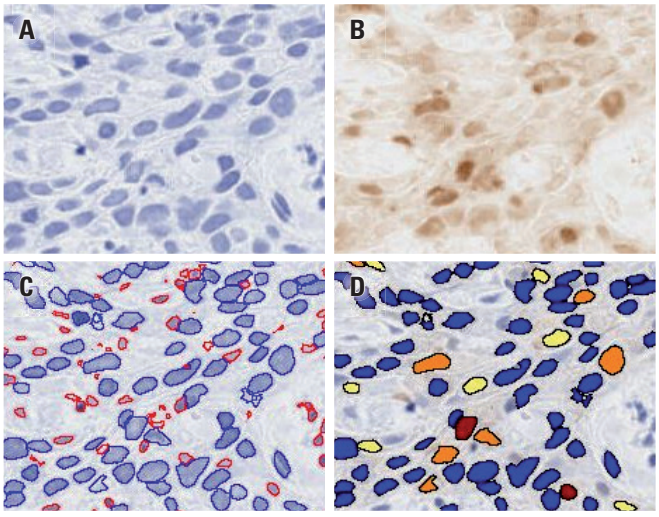

Figure 1: *Tuning the Aperio Nuclear Algorithm to detect nuclei in tissue sample. Images A & B show detection of nuclear counterstain and positive stain (in this case, Hematoxylin and DAB). Image C illustrates automatic detection of nuclei by the algorithm, as well as user selection of detected nuclei to exclude (red) based on size and shape. Image D shows final detection of nuclei and 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 frequently 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. For example, 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 as well as a number of other markers of tumor progression, and results supported the hypothesis that the COX-2 pathway promotes cancer development. The same group later performed a further study to examine COX-2's specific role in DCIS lymphatic metastasis[5], using the same image analysis methodology.

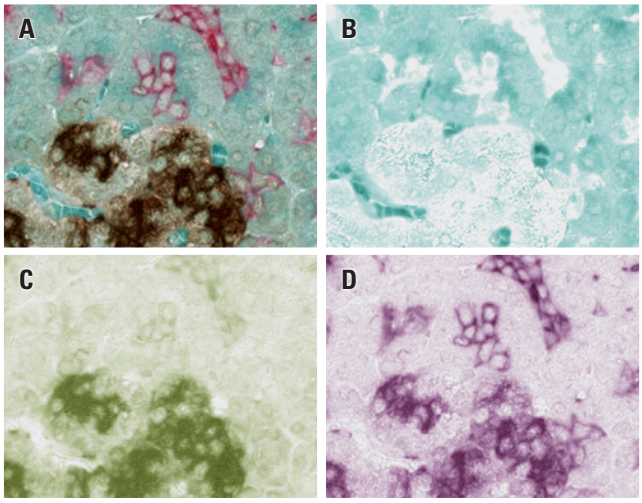

Figure 2: *Separation of trichrome staining using the Aperio Color Deconvolution Algorithm. Image A shows the original tissue, while Images B, C & D show the location of each stain (Crystal Light Green, DAB and Fast Red). As well as visual separation, the Color Deconvolution Algorithm will provide information on area and intensity of staining for each individual stain.*

Cellular proliferation and apoptotic cell death pathways are extensively studied in breast cancer research when trying to elucidate 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. For example, 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 Aperio algorithm to measure TUNEL positive nuclei, capitalizing on the ability of the algorithm to be flexibly tuned for different biomarkers. Other studies have utilized the Aperio Positive Pixel algorithm to quantify the expression of a wide range of biomarkers in pathogenesis etiology, 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 near surrounding tumorous tissue in 118 patients, separating out epithelium, adipose tissue, non-fatty stroma and glass. They first trained Aperio GENIE on a subset of slides, and compared these results with a pathologist read of both the glass slide and the digital slide image to assess performance. The results showed strong correlation between all three methods, and the authors chose to utilize 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".*

Recently Pang *et al.*[11] used several Aperio algorithms to quantify various breast tissue attributes in women at high risk of breast cancer. The 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 Microvessel Analysis algorithm was used to quantify angiogenesis, with vascular tissue stained by CD31, while the Nuclear algorithm was used to quantify a number of immuno-histochemical markers, include ER-alpha, ER-beta, PR, and Ki-67. In this study, image analysis was used to quantify a number of different attributes, contributing to a detailed view of the tissue phenotype.

PROGNOSTIC INDICATORS

Identification of markers that can act as indicators of outcome for patients is a vital step towards development of personalized medicine, and automated image analysis tools can be utilized to quantitatively identify these biomarkers.

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; however 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 later performed a larger validation 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, but that *"the introduction of digital imaging devices and computer-assisted image analysis has provided a major advance towards quantitative description of IHC [immunohistochemistry] signals".* In the validation study they used the Aperio GENIE tool to differentiate tumor and stromal regions in tissue, allowing them to accurately and automatically identify the correct cohort of cells, followed by using the Positive Pixel algorithm to measure expression of survivin only within 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 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

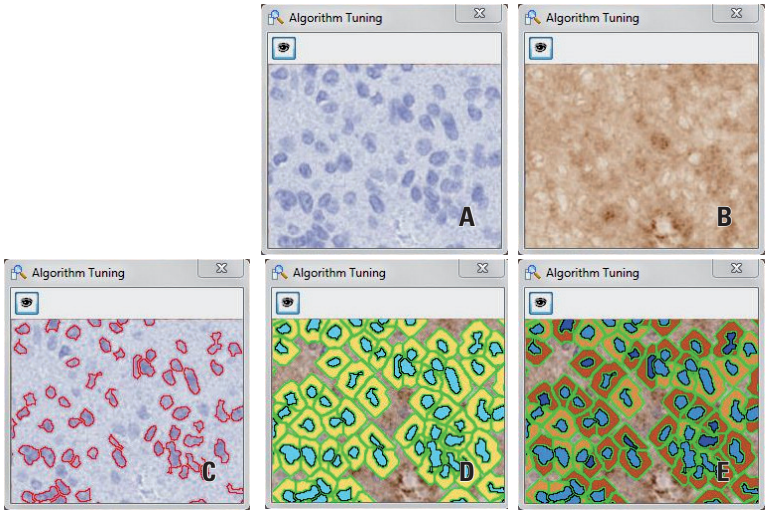

Figure 3: *Tuning the Aperio Cytoplasmic Algorithm. Images A & B show detection of nuclear counterstain (Hematoxylin) and positive stain (DAB). Image C illustrates automatic detection of nuclei by the algorithm, while Image D shows user-defined cytoplasmic separation, i.e. the distance from the nucleus to be classified as cytoplasm. Image E shows classification of cellular cytoplasmic staining, the intensity of the cytoplasm is denoted by a variation from brown to yellow, and the nuclei staining is illustrated by light to dark blue.*

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 could quantity intensity of RRM2 staining specifically in the cytoplasmic compartment of the tumor cells. In a 2012 study[16], 72 patient samples were used 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 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. A 2014 study[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 and validation 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 elucidate 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

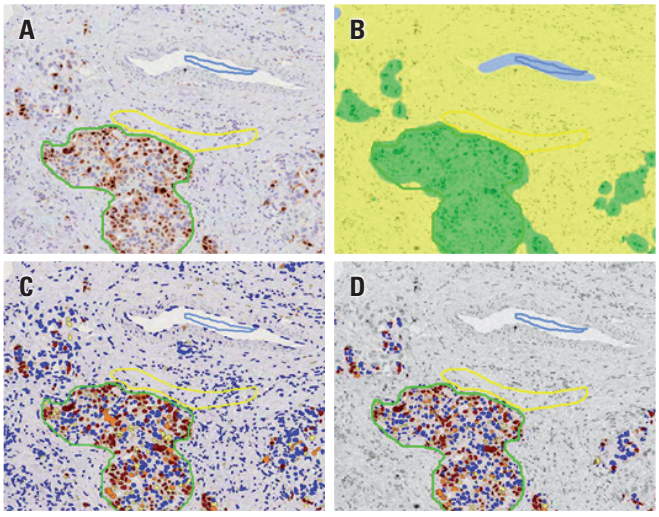

Figure 4: *Use of Aperio GENIE in conjunction with the Nuclear Algorithm to quantify biomarkers in tumor. Image A shows the original tissue sample with regions of different tissue type and glass regions annotated for Aperio GENIE training. Image B shows the tissue after Aperio GENIE training, with tumor (green), stroma (yellow), and glass (blue) identified. Image C shows analysis of the region with the 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.*

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 *"rapidly evaluate multiple endpoints in a preclinical drug efficacy study"*. They further 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.

Other studies have utilized the Aperio Nuclear algorithm to quantify multiple markers in studies of potential therapeutic targets, e.g. 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 Positive Pixel algorithm.

The Aperio GENIE tool for histology pattern recognition has also been used in studies of novel therapies, particularly when used in combination with other algorithms. For example, 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 Microvessel Analysis algorithm to measure angiogenesis in the samples. Similarly, Mignion *et al.*[23] measured tumor chemotherapeutic response, using Aperio GENIE to classify regions of tumor, necrosis and non-target tissues, and the 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.

IMAGE ANALYSIS IN THE CLINICAL ENVIRONMENT

Image analysis tools are capable of accurately quantifying biomarker staining in tissue, and have the potential to act as a valuable diagnostic aid to manual assessment of samples by pathologists. Numerous studies have investigated the potential for automated image analysis to move into daily clinical practice.

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] also 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

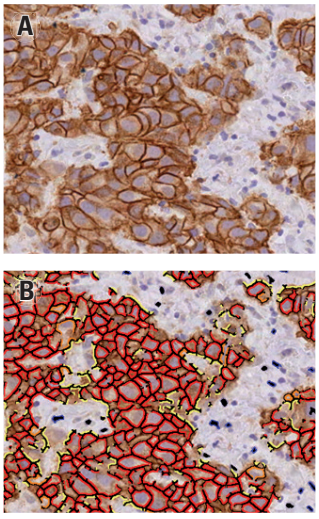

Figure 5: *Visualization of analysis by the Aperio Membrane Algorithm. Image A shows the original tissue, while Image B shows the analyzed region. The algorithm automatically detects cell membrane and based on tuning by the user, it classifies membrane areas as strongly (red), moderately (orange), or weakly (yellow) stained. The algorithm also provides output on completeness of membrane staining.*

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 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. Laurinavicius *et al.*[28] used similar methodology in their image analysis, employing Aperio GENIE in combination with the 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 use of image analysis *"to obtain more accurate, reproducible and quantitative results".* Their conclusions described image analysis as *"an efficient exploratory tool clarifying complex interdependencies in the breast cancer carcinoma IHC profiles".*

Studies have also examined image analysis as a tool for quality control in the histopathology laboratory. For example, Laurinavicius *et al.*[29] looked at use of digital image analysis for quality control of staining in clinical practice, using Aperio GENIE to identify tumor regions, and the Aperio Membrane algorithm to quantify HER2 expression within those tumor regions, across different staining batches. They found that image analysis was able to detect staining drift that was not identified during conventional microscope review, making a potentially valuable tool for quality control of IHC staining. Another study by Cardiff *et al.*[30] examined the effect of different tissue fixatives on morphology and immunohistochemistry, in human and mouse breast tumors treated with a variety of different fixatives. They used Aperio image analysis tools to evaluate a number of IHC markers, as well as shrinkage of tissue in tumor samples. The authors explained that their choice of quantitative image analysis (QIA) was *"to provide more precise data, which should be reproducible in independent laboratories",* and that while the differences in tissue morphology were *"readily apparent upon empirical inspection by pathologists…the magnitude of variation is more accurately documented by QIA".*

Validation studies, such as those discussed here, support the potential of image analysis as a quantitative tool to aid pathologist interpretation. Based on this kind of research, standardized image analysis algorithms approved for clinical practice, e.g. the Aperio eIHC tool for quantitation of ER, PR and HER2, will increasingly become the norm, helping to improve patient stratification for application of personalized medicine.

CONCLUSION

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

This review outlines the broad 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, while clinical studies and appropriate regulatory approvals are helping to bring image analysis tools into the clinical environment. Aperio tools are the most widely referenced in peer reviewed publications worldwide, with over 290 publications referencing Aperio image analysis algorithms, demonstrating their flexibility and reliability for quantitative and semi-quantitative research image analysis.

TRUNCATED INDICATIONS FOR USE:

The Aperio ePathology eIHC IVD System is an automated digital slide creation, management, viewing and analysis system. It is indicated for use as an aid in the management, prognosis, and prediction of therapy outcomes of breast cancer.

The IHC HER2 Image Analysis application is intended for use as an accessory to the Dako HercepTest™ to aid in the detection and semi-quantitative measurement of Her2/neu (c-erbB-2) in formalin-fixed, paraffin-embedded neoplastic tissue. When used with the Dako HercepTest™, it is indicated for use as an aid in the assessment of breast cancer patients for whom HERCEPTIN® (Trastuzumab) treatment is being considered.

The IHC ER Image Analysis application is intended for in vitro diagnostic use as an aid to the pathologist in the detection and quantitative measurement of ER (Estrogen Receptor) in formalin-fixed paraffin-embedded neoplastic tissue.

The IHC PR Image Analysis application is intended for in vitro diagnostic use as an aid to the pathologist in the detection and quantitation measurement of PR (Progesterone Receptor) in formalin-fixed, paraffin-embedded neoplastic tissue.

For full indications for use visit www.LeicaBiosystems.com/AperioeIHC.

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