APPLICATION NOTE

Quantitative Pathology Imaging & Analysis

Author

Clifford C. Hoyt

Akoya Biosciences, Inc.
Hopkinton, MA USA

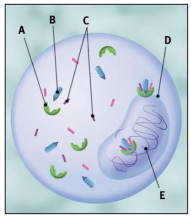
Detecting Phenotypical Subgroups in Breast Cancer using Multiplexed Protein Expression Analysis in Intact Tissue Sections

Introduction

The use of immunohistochemical (IHC) and *in situ* hybridization (ISH) methods to classify breast cancer patients is now routine. Some well-known examples are estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). In the case of HER2, it has been shown that HER2-positive patients respond favorably to Herceptin, an antibody that blocks HER2 receptors and inhibits cell-division. The HER2 success story has dramatically reduced recurrence rates and improved survival for this particular group.

The use of ER, PR and HER2 protein receptor characterization for helping identify more suitable treatments for specific groups has lead to new patient classification systems such as Luminal A (ER+, PR+, HER2-), Luminal B (ER+ and PR+, but to lesser degree of percent positivity compared to Luminal A), HER2-positive, and Basal-phenotype (ER-, PR-, HER2-).

Diagnostic and prognostic value is not likely limited to just these three receptors. Others have been discovered that are linked to breast cancer, and are currently being researched. For example, recently, a receptor called AGTR1 was discovered that is over-expressed in as many as 20% of breast cancers. AGTR1-positive cases are usually ER-positive and HER2-negative.[1]



Cell with estrogen receptors, estrogen, and helper proteins.
A Estrogen receptor
B Estrogen
C Estrogen helper proteins
D nucleus
E DNA genetic material

Reproduced from breastcancer.org

AKOYA BIOSCIENCES

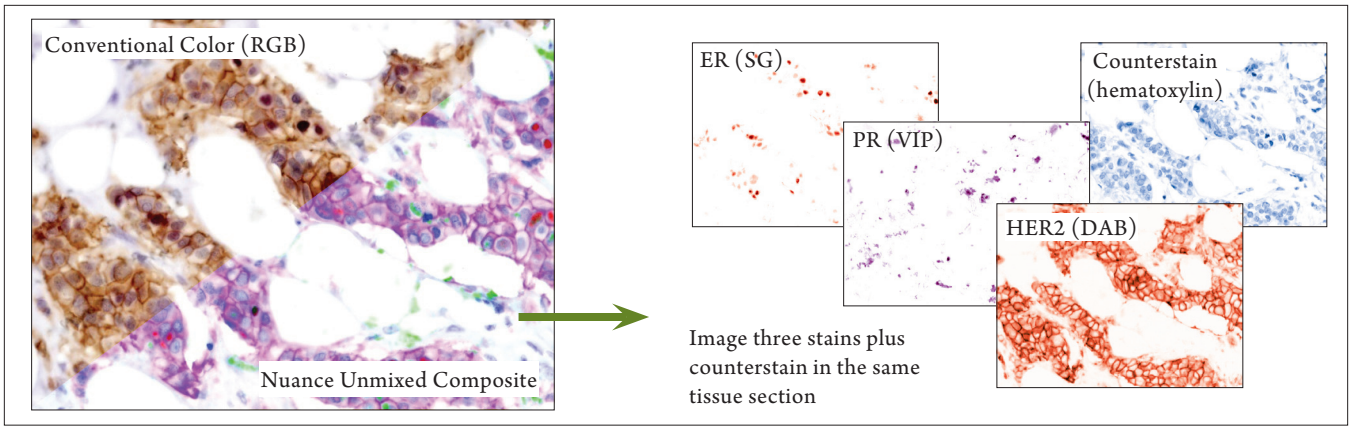


Figure 1. Breast carcinoma triple-stained for ER, PR, and HER2. (left to right): 40x color image showing what the human eye sees in the upper left and a PerkinElmer Nuance composite image in the lower right; spectrally unmixed ER (SG) image, PR (VIP) image, HER2 (DAB) image, and counterstain (hematoxylin) image. Sample courtesy Dr. Michael Feldman, University of Pennsylvania.

In addition to protein receptor-based methods for categorizing patients, researchers have identified genetic defects related to breast cancer. The most notable is BRCA1 and BRCA2 which are mutated in approximately 10% of women, and are responsible for producing proteins that repair DNA.[2] When mutated, DNA becomes vulnerable to many kinds of damage. Other mutated genes that have been linked to breast cancer are ATM, CDH1, CHEK2, p53, PTEN, and STK11. While the importance of each of these mutations to the general population is unknown, it is evident that we are at the beginning of a new era where new molecular tools help researchers and clinicians identify specific categories of patients, helping with drug development and with targeted patient care.

The Next Level of Specificity

Up until now, molecular information upon which patient classification is based has been gleaned from tissue sections, one protein at a time, from serial sections. The sections are usually stained with hematoxylin and an IHC stain, and manually assessed using the visual acuity of a pathologist.

Technology now offers the opportunity to take this approach to the next level of specificity, by providing a more quantitative measure of protein expression, and the ability to automatically assess more than one protein at a time, on a cell-by-cell basis, in intact tissue sections. Akoya's Nuance® multiplex biomarker and Vectra® quantitative pathology imaging systems and inForm advanced image analysis software provide the ideal tools for revealing this enabling information.

Precisely Characterize Stain Intensity

It is becoming apparent that characterizing the various levels of stain intensity, versus simply determining whether cells are positive or negative, reveals valuable information related to the state or progression of disease. The commonly used H-score uses four levels of stain intensity instead of two (positive and negative) as a basis for a calculation on stain intensity, and many believe that 10 levels of gradation provide even further specificity about clinical outcome.[3] Image analysis software with appropriate tools for utilizing optical density is critical to reliable image-based methods for assessing protein expression.

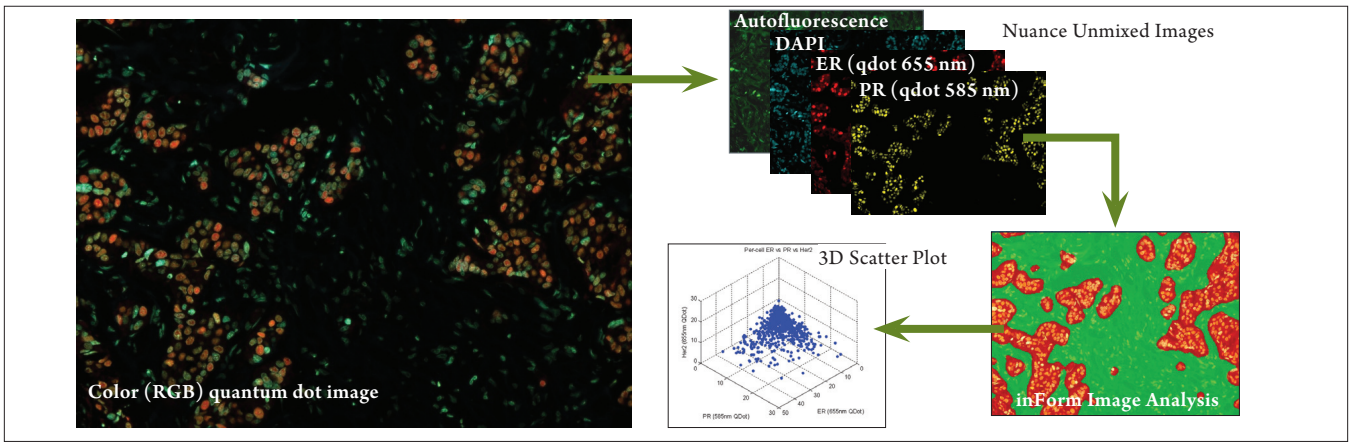


Figure 2. Breast carcinoma stained with quantum dots targeting ER, PR, and HER2. (clockwise from left): 20x color image showing what the human eye sees; spectrally unmixed autofluorescence signal; unmixed nuclei counterstain (DAPI); unmixed estrogen receptor (ER, qdot 655 nm) image; unmixed progesterone receptor (PR, qdot 585 nm) image; inForm pattern-recognition image analysis to extract data; and per-cell scatter plot showing ER, PR, and HER2. Samples and images courtesy Oncology Translational Laboratories, Novartis Institutes for Biomedical Research, Cambridge, MA, U.S. and British Columbia Cancer Agency, Vancouver BC, Canada.[5]

Detect Co-Expression in Intact Tissue

Serial sections and visual scoring give numbers that represent the average of all signals across the sample. A pathologist then visually scores the 'intensity' of the signal and estimates how many cells in a tumor are positive, to provide a 'percent positivity' score. However, when more than one marker is used per tissue section, it is very hard – sometimes impossible – to distinguish overlapping markers. The human eye is inadequate when it comes to distinguishing spatially overlapping colors, especially when one is much darker than the other.

A significant opportunity is missed by staining for only one protein at a time: if multiple proteins are stained on a tissue section, multiple positivity – co-expression – can be detected on a per-cell basis with in the same tissue section. Co-expression percent positivity could help detect sub-groups, possibly leading to even more targeted, and more effective treatments and therapies.

Multispectral Imaging Separates Multiple Markers in the Same Tissue

Quantitative, independent, and specific multi-label IHC staining protocols have been developed that, in conjunction with easy-to-use multispectral imaging systems and advanced learn-by-example software can greatly accelerate clinical and pre-clinical studies.[4] No special stains are required.

Akoya's Nuance multispectral imaging system captures and distinguishes multiple labels, such as ER, PR, HER2 and others in breast cancer biopsies. Multispectral imaging captures information from multiple wavelengths instead of simply the red, green, and blue that our eyes see, and enables the disentangling of multiple-colored protein labels even when they are spatially overlapping, something that conventional techniques and the human eye cannot do as well. The Nuance system is equally effective with multiple fluorescent probes in immunofluorescence (IF), unmixing signals from each other, as well as from ubiquitous autofluorescence. Quantum dot labeling in particular lends itself to multiplexed assays because of the distinct spectral signatures and resistance to photobleaching.[5] The Nuance system is perfect for studies where manual slide-handling and image acquisition are not prohibitive.

The powerful learn-by-example image analysis algorithms in Akoya's inForm® advanced image analysis software can also be can also be used to differentiate relevant tissue regions (e.g., malignant and normal epithelia, stroma, necrosis, etc.) and segment cellular compartments (nuclei, cytoplasm, and membrane) to allow for detailed, spatially resolved multiparameter quantitation.

Akoya's Vectra quantitative pathology system can acquire images and analyze 200 slides at a time once it has been trained with a small number of representative images. Like the Nuance system, it can be used for both brightfield and fluorescence imaging. Figure 3 shows inForm-segmented and analyzed images from a 712-core tissue microarray (TMA) – 356 patients represented in duplicate – taken by a Vectra system. The first dataset was stained for ER, PR, and HER2, with a counterstain of hematoxylin, and the second stained for PR, HER1, and HER2, with a counterstain of hematoxylin. Single-stain data for comparison was obtained from previously stained and analyzed TMAs. IHC signals were spectrally unmixed and isolated from each other and the hematoxylin counterstain. Machine-learning-based automated image analysis was performed by the inForm software program to locate cancer cells, segment subcellular compartments, and extract the IHC signals on a per-cell basis. Relative stain intensities on a per-cell basis were then analyzed with a commercial software package.[4]

Fast and Accurate

In the example shown in Figure 3, multispectral 20x images obtained of each TMA core were acquired and spectrally unmixed at a rate of three cores per minute. Automated image analysis, using algorithms that were developed by end-users in under 2 hours, took approximately 10 seconds per core, segmenting cancer-containing regions and extracting signals from relevant cell compartments.

Concordance (r) between visual scores of single stain sections and the semi-automated scores of triple-stained samples indicate equivalency (r values ranging from 0.80 to 0.90), thus validating that multiplexed IHC faithfully reflects data obtainable with single-stains.[4] This example demonstrates that multiplexed staining and detection, coupled with flow-cytometry analysis tools can be used to explore multiple protein expression patterns on a cell-by-cell basis, something that cannot be accomplished with serial single stains. Together, the innovative Nuance and Vectra multispectral platforms and groundbreaking inForm software can capture cellular and sub-cellular expression details in an intact tissue architectural context.

Vectra and inForm Multiple-Marker 712-Core Study

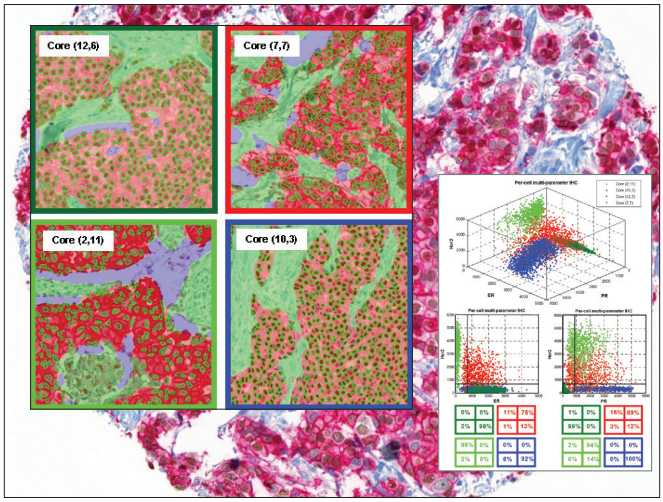


Figure 3. TMA core study using automated Vectra system and inForm image analysis software. inForm-segmented images are shown over a brightfield IHC image of a typical tissue core. Samples courtesy British Columbia Cancer Agency, Vancouver BC, Canada.

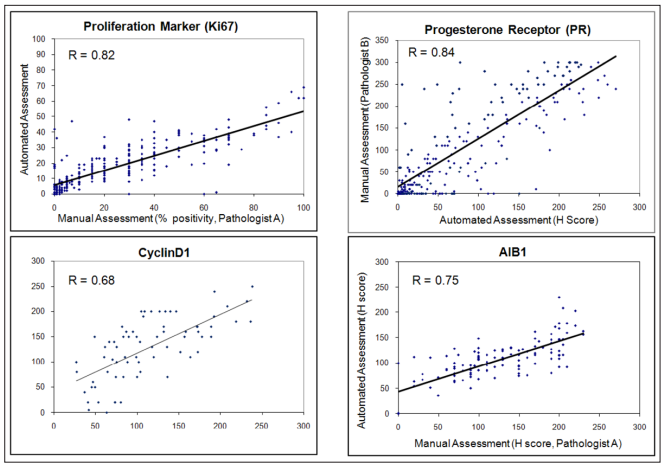


Figure 4. Concordance between automated and visual scores in study. The results from the automated, quantitative measurements performed on spectrally unmixed stain signals by Akoya's inForm were compared with visual scoring performed by pathologists on single-stained serial sections. R-values of .68 to .84, were typical, showing equivalency. Data courtesy Oncology Translational Laboratories, Novartis Institutes for Biomedical Research, Cambridge, MA, U.S.[6]

Table 1. Some breast cancer biomarkers being explored today. A more accurate assessment of the disease state may be made by simultaneous measurement of multiple biomarkers, representing various key processes that allow or drive tumor growth.

Biomarker Category	Example Biomarkers
Breast Tumor Type	ER, PR, HER2/neu
Apoptosis	Fax, FasL, TRAIL receptors, activated caspases, pAKT, Survivin, MCL-1, Bcl-2
Cell Cycle Control	p53, p21, p27, p16, Cyclins D1, E
Signal Transduction	STAT1, 3 and 5, c-kit, HSP90
Therapy Responses	MDR1, GST-it, pSR, HER2/neu
Adhesion	E-cadherin, J3-catenin, CD44, CD24, Claudin-1
Migration	CXCR4
Angiogenesis	VEGF, Flt-4, HIF-1a
Proliferation	Ki-67
Immune Response	CD3~, PD-L1, Tregs, Macrophages, Cytokines
Inflammation	NF-KB, COX2, CSF-1R

References

1. AGTR1 overexpression defines a subset of breast cancer and confers sensitivity to losartan, an AGTR1 antagonist. *Proceedings of the National Academy of Science of the United States of America,* June 1, 2009, Arul M. Chinnaiyan, University of Michigan Comprehensive Cancer Center, *et al.*

2. Memorial Sloan-Kettering Cancer Center, Breast/overian cancer website: www.mskcc.org/mskcc/html/8623.cfm

3. Quantitative, fluorescence-based in-situ assessment of protein expression. *Methods Mol Biol.* 2009;520:163-75, Moeder CB, Giltnane JM, Moulis SP, Rimm DL, Department of Pathology, Yale University School of Medicine, New Haven, CT.

4. Molecular epidemiology of clinical tissues with multi-parameter IHC, AACR 2008 Poster 237; J Ruan*, T Hope*, J Rheinhardt**, D Wang**, R Levenson*, T Nielsen‡, H Gardner**, C Hoyt*.

   *PerkinElmer, Inc., MA, U.S.

   **Oncology Translational Laboratories, Novartis Institutes for Biomedical Research, Cambridge, MA, U.S.

   ‡British Columbia Cancer Agency, Vancouver B.C.

5. Elucidating signaling networks in clinical tissues with multispectral imaging, USCAP 2009 Poster 213; T Hope*, D Fletcher-Holmes*, D Wang**, T Nielsen‡, H Gardner**, C Hoyt*.

   *PerkinElmer, Inc., MA, U.S.

   **Oncology Translational Laboratories, Novartis Institutes for Biomedical Research, Cambridge, MA, U.S.

   ‡British Columbia Cancer Agency, Vancouver B.C.

6. Multiplexed immunohistochemical quantitative molecular phenotyping via multispectral imaging and automated segmentation, San Antonio Breast Cancer Symposium 2007 Poster; C Hoyt*, K Gossage*, T Hope*, R Levenson*, R Bandaru**, H Gardner**.

   *PerkinElmer, Inc., MA, U.S.

   **Oncology Translational Laboratories, Novartis Institutes for Biomedical Research, Cambridge, MA, U.S.

PerkinElmer's Tissue Imaging Portfolio	Nuance® multispectral imaging systems for quantitative, multiple-marker analysis in immunohistochemistry and immuno-fluorescence microscopy.	Vectra® slide analysis system for automated high-throughput slide imaging and analysis, the world's only multispectral fluorescence- and brightfield-capable system.
Acquisition	Manual	Automated*
Imaging Mode	Multimodal Brightfield Immunohistochemistry (IHC) and Immunofluorescence (IF)	
Optical Imaging	Mounts to any existing microscope	Integrated
Wavelength Range	420-720 nm or 450-950 nm	420-720 nm
inForm Software	Optional	Included

*maximum capacity of 200 slides

For more information, please visit our website at www.akoyabio.com

Akoya Biosciences, Inc.
68 Elm Street
Hopkinton, MA 01748 USA
P: (+1) 855-896-8401
www.akoyabio.com

AKOYA BIOSCIENCES

For a complete listing of our US offices, visit www.akoyabio.com.
Copyright © 2019, Akoya Biosciences, Inc. All rights reserved.