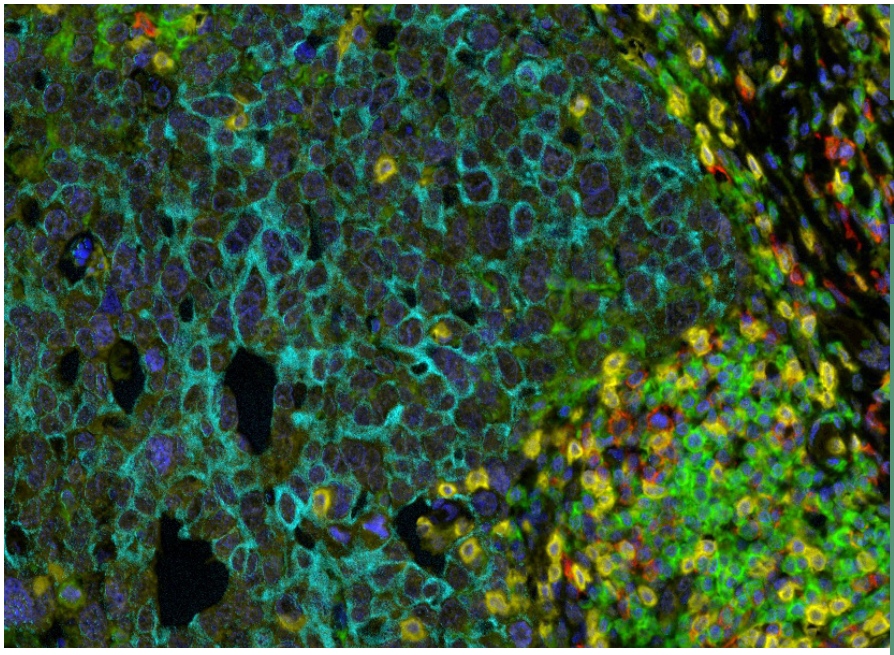

Pseudo-color composite of a breast cancer section, labeled for killer T cells (CD8, yellow), helper T cells (CD4, green), B cells (CD20, red), epithelial cells (cytokeratin, aqua) and DAPI (blue).

APPLICATION NOTE

Multiplex Biomarker Imaging

Highlights

- Immune response revealed through the characterization of infiltrating and stromal lymphocytes in FFPE tissue
- Multiplex staining with primary antibodies from the same species, enabled by covalent labeling with fluorophore conjugated tyramide
- Rapid automated analysis of whole sections using Vectra
- Quantitative results from breast cancer T and B cell panel (CD4, CD8, CD20, cytokeratin, DAPI) using inForm

Cancer Immunology: Capturing Immune Status in FFPE Sections

Introduction

Recent successes with experimental drugs targeting PD-L1 and PD-1, and adoptive immunotherapy are two examples of the tremendous potential to leverage the host's immune system to fight cancer. However, interactions between host cells, invading tumor cells and immune cells are quite complex, and are not adequately characterized by conventional single-stain immunohistochemical staining of tissue sections or by flow cytometry of disaggregated fresh tissues. In particular inflammatory cells can operate in conflicting ways, with both tumor-supporting and tumor-killing subclasses. The balance between the conflicting inflammatory responses in tumors is likely to prove instrumental in prognosis and, quite possibly, in therapies[1]. Capturing the spatial arrangements of immune, normal and malignant cells in intact tissue sections, to accurately characterize immune status, is made possible with highly multiplexed immunofluorescence labeling and multispectral imaging, offering opportunities for new assays to guide immunotherapy approach, and to monitor response. This application note describes recent advances towards an integrated approach combining highly multiplexed labeling (up to 10 labels), automated staining, imaging-based analysis to capture expression and contextual information, and data mining.

PerkinElmer®
For the Better

Multiplexed staining is accomplished by use of tyramide signal amplification (TSA) for covalent deposition of fluorophores followed by microwave treatment (MWT) to remove primary and secondary antibodies. Labels are added sequentially using a repetitive process that is simple relative to typical multicolor immunohistochemical methods. This approach allows use of any number of unlabeled primary antibodies from the same species in multiplexed assays. Labeling is specific, robust and balanced and the workflow is amenable to routine implementation.

In this study, the capability is demonstrated on control samples (tonsil) and a cohort of clinical breast cancer samples, by comparing automated and visual assessments, to perform a method proof-of-concept. However, this general methodology is extendible to a whole range of markers (up to 10 in a panel) and can combine both immune markers and other cancer, cell signaling or tumor-microenvironment markers.

Methods

A multiplexed assay for tumor-inflitrating lymphocytes for breast cancer was tested and validated on de-identified excess clinical samples, including a tonsil sample for method development, and applied to four de-identified breast cancer samples from the University of Pennsylvania Hospital. The staining approach consists of serial application of TSA-amplified immunofluorescence labels for CD4, CD8, CD20, cytokeratin, and a DAPI counterstain. Prior to immunofluorescence labeling, all four antigens are retrieved with a single microwave step. Each labeling cycle (shown in Figure 1) consists of application of a primary antibody, a secondary antibody conjugated to horse radish peroxidase (HRP), and TSA conjugated to a fluorophore. After each TSA-fluorophore conjugate is applied, the sample is processed with the microwave again to strip primary and secondary antibodies, leaving TSA-fluorophore constructs which are covalently bound and very resilient to microwave exposure.

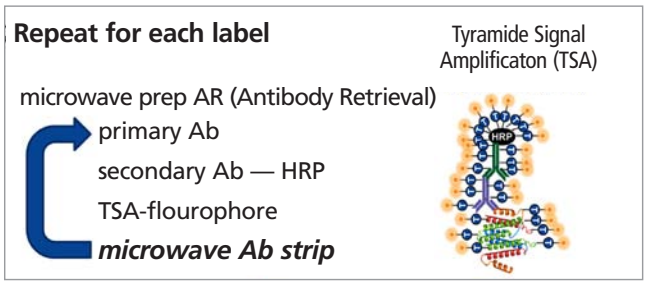

Figure 1. Same-species serial TSA immunofluorescence labeling

The fluorophores used are shown in Table 1. Because antibodies are stripped with each cycle, species interference issues are avoided, and all antibodies can be of the same species (e.g., rabbit-monoclonal antibodies), as is the case in this assay. Detailed staining protocols are available upon request.

Image acquisition and analysis

- Samples were imaged using the Vectra® multispectral slide analysis system from PerkinElmer to automatically acquire 25 fields of interest per slide. The automated workflow consists of a 4x survey of the entire slide involving the capture of a whole-slide 4x image, automated pattern recognition detection of tumor areas, and a sampling algorithm to select the 25 20x fields for multispectral imaging and analysis only from areas of interest on the sample.
- The multispectral images were analyzed using inForm® Tissue Finder™ pattern recognition software from PerkinElmer to:
  - spectrally unmix the DAPI, CD4, CD8, CD20, and cytokeratin signals and remove tissue autofluorescence;
  - segment tissue into regions of tumor and stroma;
  - and phenotype cells into categories of cancer cells, killer T cells, helper T cells, and B cells.
- Cell phenotype maps were produced from the cell segmentation data retaining spatial arrangements.
- All imagery was then carefully assessed by a pathologist for segmentation and cell phenotyping accuracy, and for distinguishing inter- versus intra-epithelial cells.

Table 1. Label-fluorophore conjugation

Marker	Cell Type	Fluorophore
CD4	Helper T cell	Fluorescein
CD8	Killer T cell	Cyanine 3
CD20	B cell	Cyanine 5
Cytokeratin	Epithelial cell	Coumarin
Counterstain	Nuclei	DAPI

Imagery and data of tonsil samples used in assay development and optimization are shown in Figures 2 and 3.

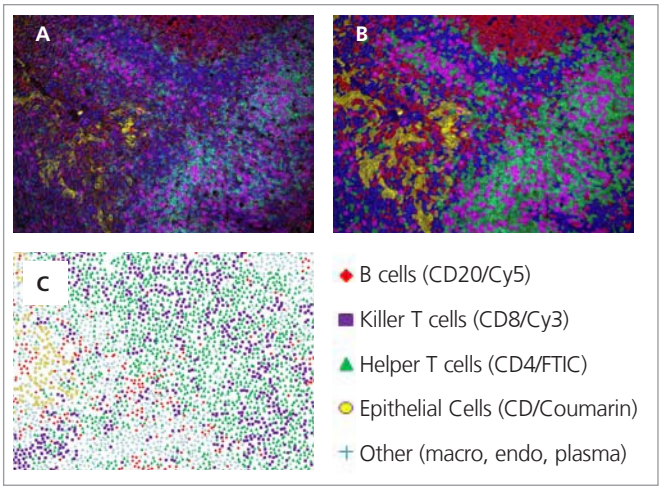

Figure 2. (A)Unmixed composite of a multispectral image from a tonsil biopsy section. (B) Image with inForm automated segmentation finding B cells (CD20+) in red, Killer T cells (CD8+) in purple, Helper T cells (CD4+) in green, Epithelial cells (CK) in yellow, and other cells in blue. (C) A cell phenotype map created from the spatial information obtained in the segmentation data.

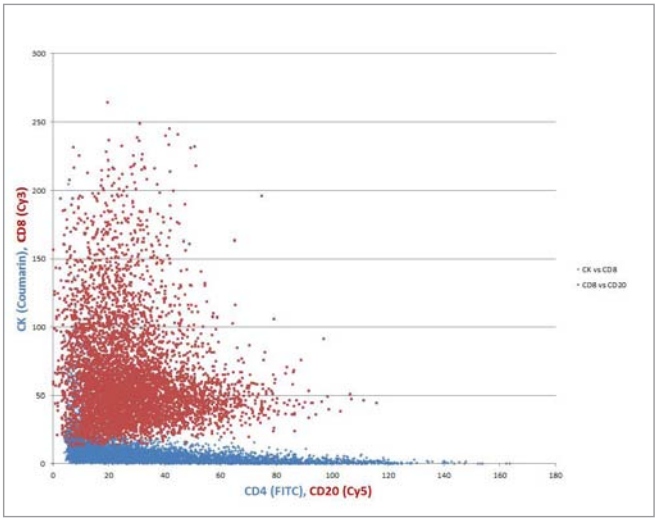

Figure 3. Per-cell expression levels from tonsil biopsy analysis plotting CK vs CD8 and CD8 vs CD20.

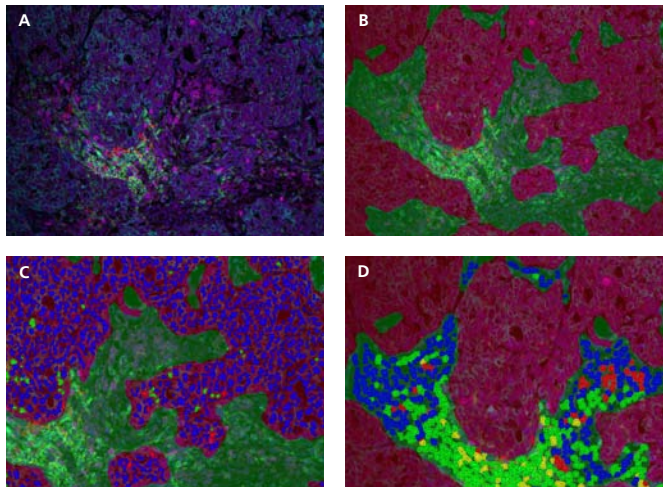

Figure 4. Example 20x field from one of the four breast cancer samples. (A) Unmixed composite image from multispectral data separating CD4, CD8, CD20, cytokeratin, and DAPI from each other and from autofluorescence. (B) Image after inForm analysis with automated tissue segmentation separating tumor (in red) from stroma (in green), (C) tumor cells segmented and scored with CD8+ only cells shown in red, CD4+ only cells shown in green, cells double positive for both CD8 and CD4 shown in yellow, and double negative cells in blue, and (D) same as in (C) but for cells in stroma regions.

Table 2. Results from image analysis of breast cancer imagery

Killer T cells (CD8+)		
Case #	% in tumor	Density (/sq mm)
#21	8.1%	574
#22	0.6%	31
#23	1.7%	99
#24	6.1%	482
Helper T cells (CD4+)		
Case #	% in tumor	Density (/sq mm)
#21	0.9%	67
#22	0.0%	0
#23	0.4%	25
#24	0.0%	10

Results

Labeling of tonsil samples demonstrated specificity and sensitivity estimated by a pathologist to be greater than 95% accurate (Example 20x field shown in Figure 4). On breast cancer samples, comparable accuracy was achieved. More importantly however, in the breast cancer samples, distinguishing inter- and intra-epithelial immune cells was estimated to be nearly perfect, despite significant inflammation in non-epithelial areas immediately adjacent to tumor, in some samples. Tumor T cell, killer/CD8 and helper/CD4 densities are shown in Table 2.

Conclusions

The approach shows reliable detection of phenotypes and accurate segmentation of tumor and stromal regions, to accurately assess how immune cells are interacting with the tumor mass, such as tumor infiltration to combat the cancer. These results support the feasibility of a practical and viable clinical workflow, in which immune response assessment is automated by computer and results are reviewed by pathologists to assure data quality.

The methodology shown in this example can be extended to a whole range of immune cell and other cancer markers and can be expanded to up to eight or nine fluorophores. In the field of cancer immunology in particular this is of great importance in exploring the role of immune cells in the tumor microenvironment.

References

[1] Hanahan, D. & Weinberg, R.A. Hallmarks of cancer: the next generation. Cell 144, 646-674 (2011).

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

PerkinElmer®

For a complete listing of our global offices, visit www.perkinelmer.com/ContactUs

011313_01