Quantitative Multiplex Immunofluorescence: A Powerful Tool for Oncology Therapeutic Development

Darren W. Davis, PhD
Senior Vice President, Precision for Medicine

Jesus Garcia, PhD
Scientific Liaison, Precision for Medicine

PRECISION
for medicine ®

Table of contents

Quantitative multiplex immunofluorescence: a powerful tool for oncology therapeutic development

Advances in our understanding of the complex, dynamic interactions between malignant tumors and the immune system have led to the development of transformative immuno-oncology therapies that leverage the body's immune system to fight cancer. These treatments, along with targeted therapies, have become integral to the management of certain types of cancer, increasing the need for tumor profiling technologies that can help predict response or resistance to different therapeutic approaches.

In this white paper, we explore the role of the tumor microenvironment (TME) in cancer progression and discuss the emerging role of quantitative multiplex immunofluorescence for tumor profiling and methods to maximize the utility of this technology.

Overview of immuno-oncology

Immuno-oncology therapeutics can be broadly categorized as:

- Immune checkpoint inhibitors, which affect molecules that prevent the immune system from attacking cancer cells

- Monoclonal antibodies, which target specific tumor antigens

- Therapeutic vaccines, which can boost or prime the immune system

- Adoptive cellular therapy, which involves removal, engineering, and re-infusion of a patient's own immune cells to enhance the immune system's anticancer response

While immuno-oncology treatments have changed the therapeutic landscape for certain cancers, most of these treatments are only effective in a subset of patients. Even when these strategies are effective, they may not result in prolonged disease control.[1] This has led to the emergence of combination strategies, as well as the search for biomarkers and tumor profiling tools, which can help predict those patients who are most likely to respond.
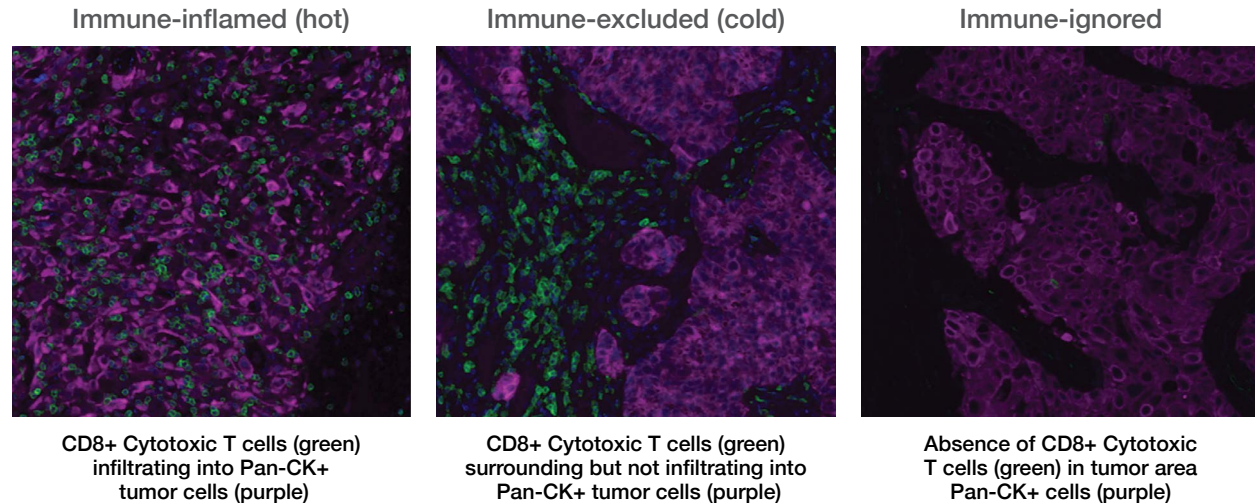
Importance of the tumor microenvironment

Knowledge of the interplay between tumor cells and the immune system is critical for the translation and development of novel cancer therapies. In solid tumors, interactions of cancer cells with their microenvironment—the mixture of cellular elements around them—can profoundly influence the probability of tumor progression, as well as the likelihood of therapeutic response and resistance.[2] A growing body of research supports the central role of the TME in multiple stages of tumor development, including local resistance, immune escape, and metastasis.[3] By secreting cytokines, chemokines, and other substances, cancer cells can functionally shape their microenvironment and reprogram surrounding cells, including immune cells. For example, certain tumor-derived molecules can inhibit T cell proliferation and survival, and tumor-induced vasculature can limit T cell migration.[4] Consequently, this reprogramming can create an immunosuppressive microenvironment that promotes tumor growth and metastasis.[5]

The level of immune suppression observed in human cancers can be categorized as (see Figure 1)[4]:

- Immune-inflamed, or hot, with homing of T cells to the tumor and an abundance of T cells infiltrating into the tumor

- Immune-excluded, or cold, with homing of T cells to the tumor but little to no T-cell infiltration into the TME

- Immune-ignored, with neither homing nor immune cell infiltration

Cancers that are most often characterized as immunologically hot include melanoma, head and neck, non-small cell lung cancer, liver, kidney, bladder, and cancers with high microsatellite instability. Cancers that are most often characterized as cold include pancreatic, ovarian, prostate, and many breast cancers.[6]

Figure 1. **Immune classification of TMEs.**

Immune-inflamed (hot)	Immune-excluded (cold)	Immune-ignored

| CD8+ Cytotoxic T cells (green) infiltrating into Pan-CK+ tumor cells (purple) | CD8+ Cytotoxic T cells (green) surrounding but not infiltrating into Pan-CK+ tumor cells (purple) | Absence of CD8+ Cytotoxic T cells (green) in tumor area Pan-CK+ cells (purple) |

Images of breast and lung cancer stained with an Immuno-Oncology panel and imaged using Precision's Multiplex Immunofluorescence workflow.

Research has demonstrated that immune checkpoint inhibitors and cytotoxic immune cells only home on and infiltrate hot, or very warm, tumors. As such, the ability to distinguish between hot and cold tumors holds therapeutic significance. It is believed that immune checkpoint inhibitors work by activating T cells that have already responded to the tumor but are being suppressed by the cancer.[6] Hot tumors contain high levels of infiltrating T cells, increasing the likelihood that they will be recognized by the immune system and trigger an immune response. Moreover, hot tumors often have a high mutation burden, with surface neoantigens that make these cancer cells more likely to be flagged as foreign by the immune system.[7]

Our current understanding is that the success of infused activated cellular therapies and treatments that work via immune cell activation relies upon:

- Proximity of the activated cells to the target tumor cells

- Effective tumor infiltration followed by activation of immune cells already residing in the tumor

Immune cells in less than warm TMEs exist in immune-suppressed surroundings. Although these immune cells may reside within the tumor, they are inactivated or suppressed, rendering the tumor as cold.

Methods for monitoring response to immuno-oncology therapies

Studies have shown that a preexisting immune landscape within the TME may have prognostic value in a number of malignancies and may be useful as a predictive biomarker of response to certain types of immunotherapy.[4] There are a multitude of methods for assessing and monitoring immune response in the TME, including

- **Flow cytometry,** which can be used for cell cycle analysis, immunophenotyping, rare event analysis, and detection of minimal residual disease, among other applications

- **Immunohistochemistry (IHC),** which is used to determine levels of protein expression

- **Chromogenic/fluorescence *in situ* hybridization (CISH/FISH),** which detects gene alterations including deletions, amplifications, translocations, and fusions

- **Next-generation sequencing (NGS),** which rapidly examines the genome and broadly detects DNA mutations, copy number variations, and gene fusions

- **Whole exome sequencing (WES),** which can be used for assessing mutational burden and its influence on antitumor immunity

- **RNA sequencing,** which is used to profile the transcriptome

- **Cytokine profiling,** which can be used to demonstrate patterns of cytokine response during immunotherapy[8]

- **Multiplex immunofluorescence,** which is used for simultaneous detection of several target proteins within a single cell, as well as examination of the spatial arrangement of cell phenotypes

From a clinical perspective, the methods used for monitoring response to immuno-oncology therapy may vary by tumor type and may depend on the type and quantity of tissue available. For example, preservation techniques such as freezing and formalin fixation have been shown to alter certain immune cell subsets, cytokine profiles, and even genomic variants, making archival tissue unsuitable for certain applications such as flow cytometry and WES.[9] Quantity of tissue available may also limit the number of assays that can be performed. It is therefore important to be able to maximize the amount of data acquired from an individual sample. Multiplex immunofluorescence has emerged as a powerful method for addressing some of the challenges inherent in tumor profiling and immune response monitoring. As a relatively new technology, multiplex immunofluorescence may not be well understood, and new applications are evolving.

Introduction to quantitative multiplex immunofluorescence

The promise of precision oncology lies in the ability to develop a comprehensive understanding of each patient's tumor and TME to individualize treatment. Thus, the utilization of precision medicine approaches in oncology therapeutic development depends on effective tumor profiling. Often, only a small biopsy sample is available for performing the wide variety of tests needed to confirm the subtype of a tumor and check for prognostic or predictive biomarkers to guide treatment. In therapeutic areas such as lung cancer, where the number of validated biomarkers

has grown, researchers and pathologists are faced with the challenge of how to test for a large set of biomarkers on a limited amount of tissue. In addition, our growing understanding of the impact of the TME on tumor progression and treatment response has led to an increased focus on examining biopsy specimens to characterize the complexities of the TME.

Quantitative multiplex immunofluorescence, hereafter referred to as multiplex immunofluorescence, is a technology that allows for simultaneous detection of multiple target proteins in the same formalin-fixed

paraffin-embedded (FFPE) tissue section or even the same cell, while preserving tumor material. Tissue multiplexing immunofluorescence capabilities allow for the detection of up to 9 markers, which can be used to phenotype and identify important cell populations. In essence, this technology not only provides information on biomarker expression levels but also increases the number of biomarkers that can be visualized at the same time (Figure 2).
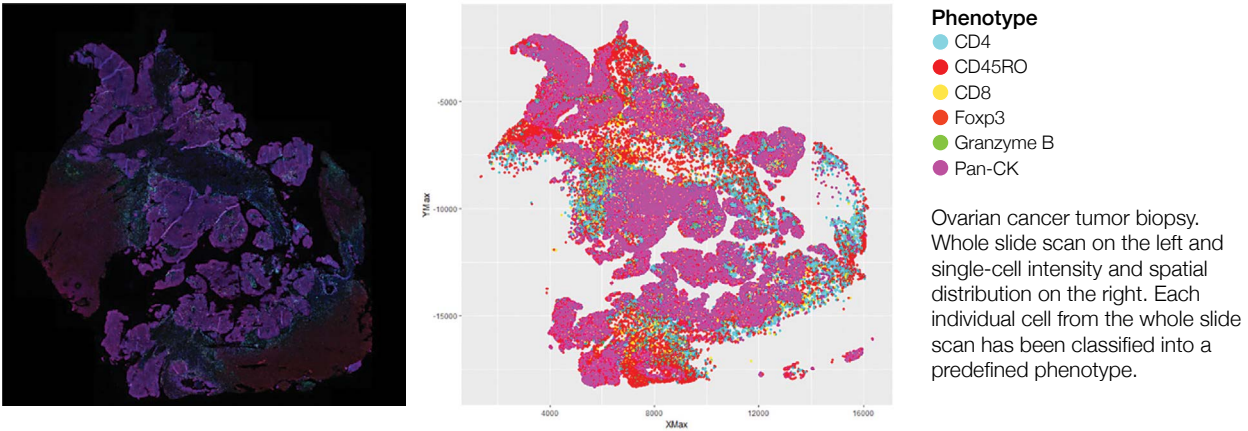
Figure 2. **Whole-slide single-cell analysis.**

Whole slide scan of breast cancer section stained with an 8-color panel. Scanning fields are represented by a green grid mask; each square in the grid is a "Region of Interest" (ROI). The included summary table shows whole slide total cell counts, individual marker counts, percentages, and mean fluorescence intensity (MFI).

Whole slide scanning enables the generation of ROIs covering the entire biopsy section to be stitched together for a comprehensive analysis.

Metric	Total Cells	CD11c	PD-L1	CD45	CD14	CD16	CD123	CD141
Counts	123245	20087	3077	34595	6562	10746	43118	7854
Percentage	NA	16.30	2.50	28.07	5.32	8.72	34.99	6.37
MFI	NA	3.21	1.21	0.75	0.09	0.05	10.72	10.77

Multiplex immunofluorescence also has the capacity to provide information on the spatial distribution and activation state of different types of immune cell populations within a sample, providing much needed insight into the TME. Using software to track each cell and its associated data, it is now possible to explore the architectural context of the TME, which can help to distinguish hot and cold tumors and stratify patients for immunotherapy (Figure 3).

Figure 3. **Single-cell detection, phenotyping, and spatial distribution from a single sample.**

Phenotype
- CD4
- CD45RO
- CD8
- Foxp3
- Granzyme B
- Pan-CK

Ovarian cancer tumor biopsy. Whole slide scan on the left and single-cell intensity and spatial distribution on the right. Each individual cell from the whole slide scan has been classified into a predefined phenotype.

Maximizing the utility of multiplex immunofluorescence

At Precision for Medicine, we have developed a digital pathology workflow that uses the PhenoImager HT™ (Vectra Polaris) Multiplex Immunofluorescence System. This system integrates multispectral imaging with automated scanning of cells *in situ* in FFPE tissue sections and tissue microarrays. Precision for Medicine has been able to maximize the capabilities of the PhenoImager HT™ (Vectra Polaris) system using a customized, 4-step workflow shown in Figure 4.

Figure 4. **Precision for Medicine workflow for tissue multiplexed profiling.**
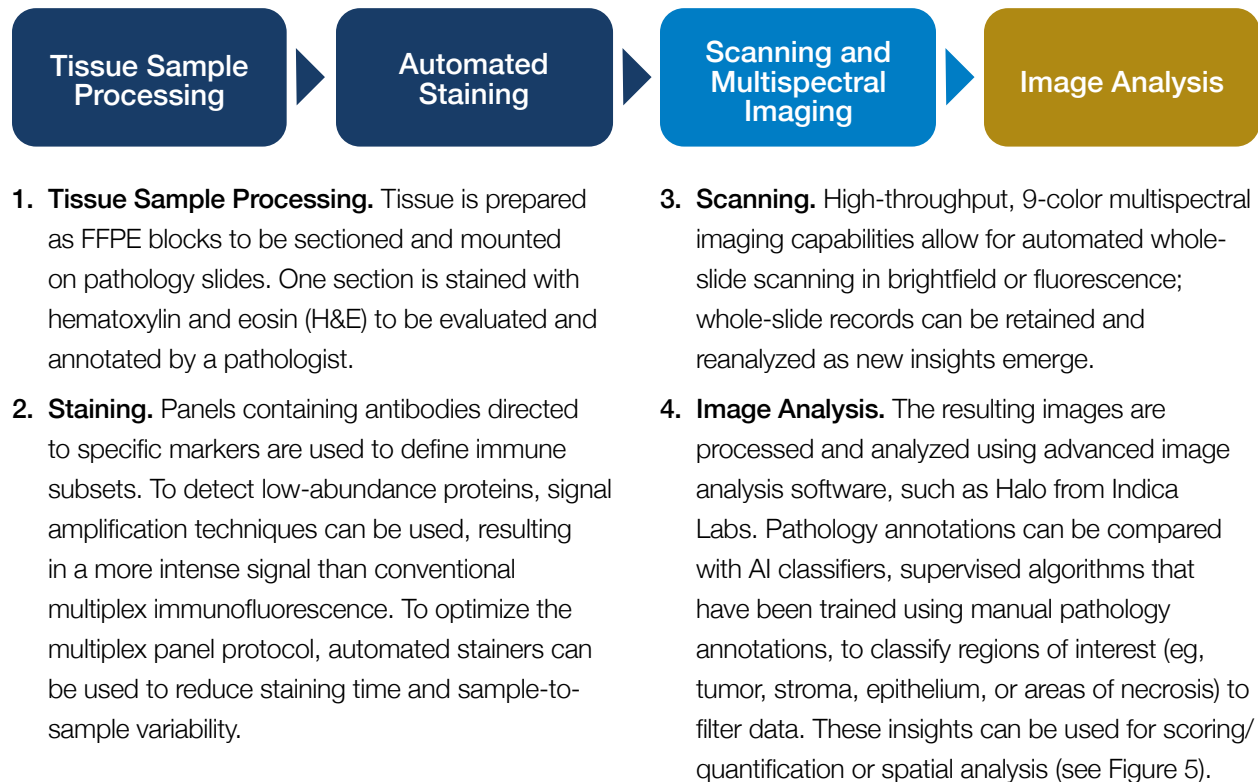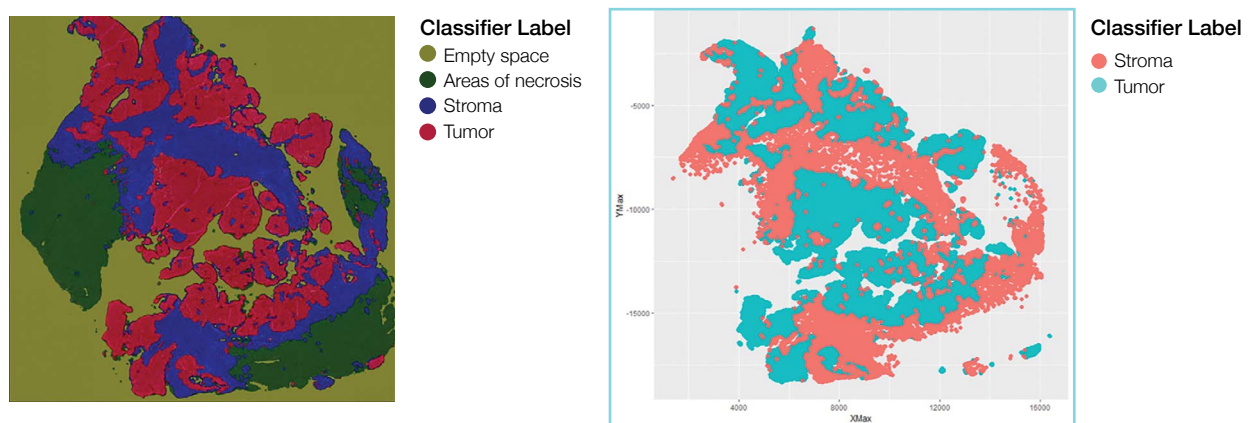
Tissue Sample Processing	▶	Automated Staining	▶	Scanning and Multispectral Imaging	▶	Image Analysis

1. **Tissue Sample Processing.** Tissue is prepared as FFPE blocks to be sectioned and mounted on pathology slides. One section is stained with hematoxylin and eosin (H&E) to be evaluated and annotated by a pathologist.

2. **Staining.** Panels containing antibodies directed to specific markers are used to define immune subsets. To detect low-abundance proteins, signal amplification techniques can be used, resulting in a more intense signal than conventional multiplex immunofluorescence. To optimize the multiplex panel protocol, automated stainers can be used to reduce staining time and sample-to-sample variability.

3. **Scanning.** High-throughput, 9-color multispectral imaging capabilities allow for automated whole-slide scanning in brightfield or fluorescence; whole-slide records can be retained and reanalyzed as new insights emerge.

4. **Image Analysis.** The resulting images are processed and analyzed using advanced image analysis software, such as Halo from Indica Labs. Pathology annotations can be compared with AI classifiers, supervised algorithms that have been trained using manual pathology annotations, to classify regions of interest (eg, tumor, stroma, epithelium, or areas of necrosis) to filter data. These insights can be used for scoring/quantification or spatial analysis (see Figure 5).

Figure 5. **AI classifier analysis.**

Ovarian cancer tumor biopsy. Pathology annotation on the left and AI classifier on the right. The AI classifier assigns a classification to every cell and can be used to filter out exclusion classes (eg, empty space/glass and areas of necrosis) leaving only those cells classified as stroma or tumor in the image on the right.

To date, Precision for Medicine has validated several immune biomarker panels across various therapeutic applications using this biomarker multiplexing platform and workflow. These panels are being used to monitor immune cell infiltration in certain cancers, psoriasis, lupus, and atopic dermatitis.

Importance of spatial biology

Research has shown that spatial characterization provides critical insight into tumors. Within the TME, it is not just the frequency or ratios of immune cells but also their proximity to suppressive elements that may predict tumor progression, response to immuno-oncology treatment, and even recurrence. There is increasing evidence that cell-to-cell topography and the resulting probability of cell-to-cell interactions can be correlated to clinical and prognostic parameters.[10] For example, a recent meta-analysis showed that multiplex immunofluorescence with spatial characterization demonstrated improved performance over other biomarker testing approaches, including gene expression profiling, tumor mutational burden assessment, and IHC, in predicting patient response to anti–PD-1/PD-L1 therapies across 10 different solid tumor types.[11]

Common spatial analyses using multiplex immunofluorescence include nearest neighbor analysis, proximity analysis, and infiltration analysis (see Figures 6, 7, and 8).
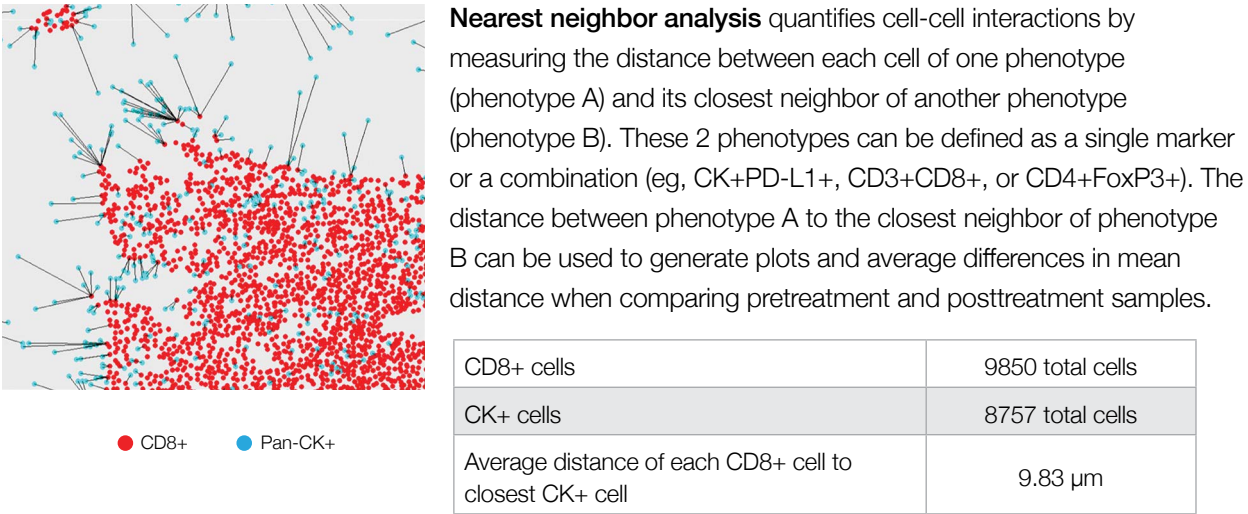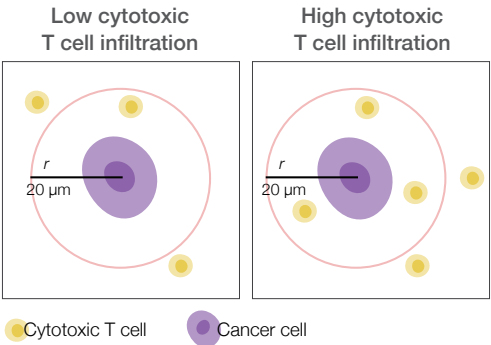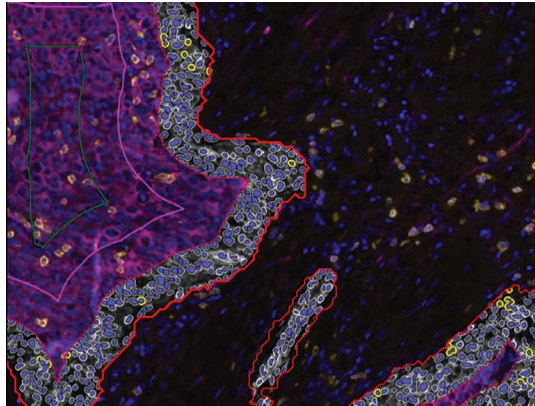

Figure 6. **Nearest neighbor analysis.**

● CD8+   ● Pan-CK+

Nearest neighbor analysis quantifies cell-cell interactions by measuring the distance between each cell of one phenotype (phenotype A) and its closest neighbor of another phenotype (phenotype B). These 2 phenotypes can be defined as a single marker or a combination (eg, CK+PD-L1+, CD3+CD8+, or CD4+FoxP3+). The distance between phenotype A to the closest neighbor of phenotype B can be used to generate plots and average differences in mean distance when comparing pretreatment and posttreatment samples.

CD8+ cells	9850 total cells
CK+ cells	8757 total cells
Average distance of each CD8+ cell to closest CK+ cell	9.83 µm

---

Figure 7. **Proximity analysis.**

Low cytotoxic
T cell infiltration

High cytotoxic
T cell infiltration

● Cytotoxic T cell   ● Cancer cell

Proximity analysis measures the interactions between different cells by counting cells within a specified radius of every cell of a given phenotype.

In this analysis, the radius of the given phenotype (cancer cell) is specified to be 20 µm and the analysis is used to quantify the number of cytotoxic T cells within the radius. The image was adapted from: Spatial computation of intratumoral T cells correlates with survival of patients with pancreatic cancer, J. Carstens et al. *Nat Commun*. 2017.

Figure 8. **Infiltration analysis.**

Infiltration analysis quantifies the number, penetration, and migration of immune cell infiltration and can be used to quantify any number of predefined phenotypes located within a specified distance from a predefined annotation. This annotation is commonly a tumor margin annotated by a pathologist. The distance can be divided into bins in a concentric fashion for semiquantitative analysis, and the width of the bins can be defined by users. Concentric areas outside the margin can also be created to analyze tumor proximity rather than tumor infiltration.

In this analysis, the red line corresponds to the tumor margin, drawn by AI-assisted manual annotation. The dotted red, pink, and green lines are software-generated concentric bins each representing approximately 50 μm in thickness.

Technologies that can be used to complement multiplex panels include highly multiplexed approaches for panel narrowing or screening and spatial or spatiotemporal gene expression.

A recent study examined multiparametric immune profiling in oral squamous cell cancer using a combination of multispectral imaging, objective assessment, and conventional IHC. This study found that, as reported in other cancer types, a high density of CD8+ T cells at the invasive margin correlated with prolonged overall survival. Interestingly, CD8+ T cell numbers on the tumor side of the invasive margin had a greater effect on survival than that on the stromal side, suggesting that relative location of immune cell infiltration is important for prognosis. This study also demonstrated that a higher number of suppressive elements—in this case, FoxP3+ and PD-L1+ cells—within 30 μm of CD8+ T cells reduced overall survival. These findings have clinical significance, particularly in a therapeutic area such as oral squamous cell cancer where risk stratification based on traditional tumor size, lymph node, and distant metastasis (TNM staging) is insufficient for
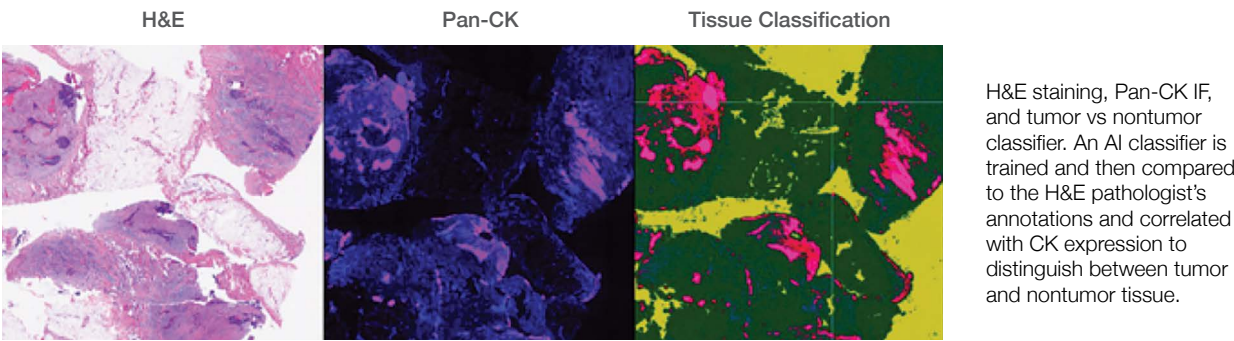
predicting prognosis and other biomarkers are needed.[10]

Just as tumors are heterogeneous, TMEs exhibit heterogeneity as well. Comprehensive evaluation of the composition and distribution of—and spatial relationships among—immune cells and other elements in the TME will help researchers better understand the cells' roles in tumor development and progression. A more detailed understanding of the spatial biology of individual tumors may also shed light on the critical distinctions between hot and cold tumors, enabling the development of strategies to turn cold tumors hot.

Case study: patient stratification based on infiltration analysis

In a recent study, Precision for Medicine demonstrated the ability of its profiling pipeline to detect patients who might exhibit improved therapeutic response based on analysis of tumor infiltration by CD4+ and CD8+ cells. In this study, tissue was first classified as tumor or nontumor (see Figure 9).
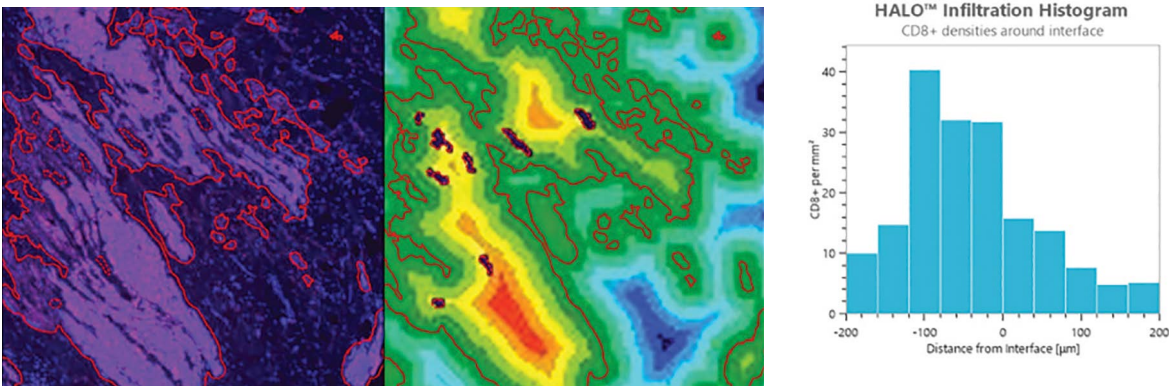
Figure 9. **Tissue classification for infiltration analysis.**

| H&E | Pan-CK | Tissue Classification |

H&E staining, Pan-CK IF, and tumor vs nontumor classifier. An AI classifier is trained and then compared to the H&E pathologist's annotations and correlated with CK expression to distinguish between tumor and nontumor tissue.

Once the tissue was classified, an infiltration analysis tool was used to define regions of quantification of immune cell infiltration (see Figure 10). Deeper insight into the level of immune cell infiltration allows for distinction between hot and cold tumors and can be used in clinical trials to determine eligibility and to stratify patients who are most likely to respond to immunotherapy.

Figure 10. **Immune cell infiltration analysis and histogram.**

An infiltration analysis tool is used to define concentric regions inside and outside the tumor margin for quantification of infiltration. These data can be used to generate an infiltration histogram, in this case showing CD8+ cells/mm^2 per region. In the histogram, 0 represents the tumor margin. Negative values are distances inside the tumor. Positive values are distances outside the tumor.

Conclusion

Immunotherapy is an ongoing revolution in cancer treatment that requires a deeper understanding of the TME. Multiplex immunofluorescence techniques have emerged as powerful tools for studying immune cells in context, providing valuable insight into the TME, and creating opportunities to identify potential biomarkers and new therapeutic targets.[12] With its ability to detect multiple markers simultaneously and define the spatial relationships among cells, multiplex immunofluorescence is expected to play an increasingly important role in both immune profiling and translational research.

At Precision for Medicine, we believe that multiplex immunofluorescence is an important tool in biomarker-driven therapeutic development in immuno-oncology, as well as in autoimmune and other indications such as lupus, atopic dermatitis, and psoriasis. The applications of multiplex immunofluorescence from exploratory studies to late-stage trials, will continue to expand. By combining multiplex immunofluorescence with our ApoStream® technology for isolating and enriching circulating tumor cells, we are accelerating immuno-oncology drug development using both tumor and liquid biopsies to advance our understanding of the biological correlation between cancer cells present in tumor tissue and those circulating in the blood.

References

1. Hofman P et al. Multiplexed immunohistochemistry for molecular and immune profiling in lung cancer—just about ready for prime-time? *Cancers (Basel)*. 2019;11(3):283.

2. Nature. The tumour microenvironment. Nature website. https://www.nature.com/collections/khylqkxqbr. Accessed November 20, 2019.

3. Chen F et al. New horizons in tumor microenvironment biology: challenges and opportunities. *BMC Med*. 2015;13:45.

4. van der Woude LL et al. Migrating into the tumor: a roadmap for T cells. *Trends Cancer*. 2017;3(11):797-808.

5. Hinshaw DC, Shevde LA. The tumor microenvironment innately modulates cancer progression. *Cancer Res*. 2019;79(18):4557-4566.

6. The ASCO Post. How turning 'cold' tumors into 'hot' ones may improve response to immunotherapy. February 10, 2019. The ASCO Post website. https://www.ascopost.com/issues/february-10-2019/turning-cold-tumors-into-hot-ones/. Accessed February 13, 2020.

7. Dana-Farber Cancer Institute. Enhancing immunotherapy: the race for make "cold" tumors "hot." Dana-Farber Cancer Institute website. https://blog.dana-farber.org/insight/2018/06/enhancing-immunotherapy-race-make-cold-tumors-hot/. Accessed February 13, 2020.

8. Siebert JC, Walker EB. Monitoring cytokine profiles during immunotherapy. *Immunotherapy*. 2010;2(6):799-816.

9. Wargo JA, Reddy SM, Reuben A, Sharma P. Monitoring immune responses in the tumor microenvironment. *Curr Opin Immunol*. 2016;41:23-31.

10. Feng Z et al. Multiparametric immune profile in HPV-oral squamous cell cancer. *JCI Insight*. 2017;2(14):e93652.

11. Lu S et al. Comparison of biomarker modalities for predicting response to PD-1/PD-L1 checkpoint blockade: a systematic review and meta-analysis. *JAMA Oncol*. 2019. DOI: 10.1001/jamaoncol.2019.1549. [Epub ahead of print]

12. Francisco-Cruz A, Parra ER, Tetzlaff MT, Wistuba II. Multiplex immunofluorescence assays. *Methods Mol Biol*. 2020;2055:467-495.

trials

data

labs

PRECISION
for medicine ®