SIX PLEX FOR SUCCESS
and Other Strategies for Developing Clinically Useful Spatial Signature Biomarkers

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INTRODUCTION

The key to creating more predictive biomarker tests for immunotherapy response is exploring the presence, distribution, and functional state of immune cell types in the tumor microenvironment (TME), and how they signal and interact with one another and with tumor cells [1]. Spatial signatures of the TME are defined by measuring the interactions and cellular densities of tumor and immune cells in the TME and represent an emerging class of biomarkers with demonstrated prognostic value [2, 3].

Multiplexed immunofluorescence (mIF) has been shown to be a well-suited assay methodology for measuring spatial signatures [4, 5]. Capable of accurately and sensitively revealing cell types and expressions related to immune response, mIF can be used to quantitate and localize T cells, B cells, macrophages, tumor cells, as well as complex structures comprising multiple cell types. In particular, mIF reveals ligands and associated receptors related to immune activation and suppression, of which there are multiple identified pairs, each directly linked to specific immunotherapies.

Spatial signatures can be based on the presence, absence, density, or proximity of detected cellular signals and phenotypes within a given tumor, stroma, or invasive margin. They can also be based solely on or in combination with the presence or absence of structures, such as tertiary lymphoid structures (TLSs) or unique cellular neighborhoods.

KEY TAKEAWAYS

- Multiplex immunofluorescence (mIF) has been shown to be a well-suited assay methodology for measuring spatial signatures
- Spatial signatures as highly predictive biomarkers for cancer immunotherapy has been demonstrated across a wide range of cancer types
- Six marker panels for spatial signature development are ideally suited for translation into the clinic
- The PhenoImager Solution provides the right tools and an integrated workflow for the accelerated development of spatial signatures

Faced with so many possible determinants of a spatial signature, and faced with intra- and inter-sample heterogeneity, translational researchers may find it daunting to design and optimize a spatial signature biomarker assay. Fortunately, mIF using whole-slide scanning at single cell resolution provides the right balance of multiplex detection capability, high throughput processing power, reproducibility, sensitivity, and accurate image analysis to yield reliable data across heterogeneous samples.

This white paper suggests best practices, tips, and tools for developing spatial signature biomarkers designed for fast, reproducible use in translational and, potentially, clinical research settings.

Challenges of Moving Spatial Signatures to the Clinic

In addition to the core requirements that apply to any assay platform for translational oncology research (FIGURE 1), translating a spatial signature to a biomarker assay faces additional challenges that fall into three main categories:

1. **Choosing the right analytes to measure**

   Analytes that accurately characterize the TME must reflect the identity of individual TME cell types and report key biological functions of TME components. It is important to choose analytes that can answer key questions, such as "What is the level of T cell infiltration in the tumor?", "Where are immune cells in the TME?", "Are tumor cells proliferating?", "Are lymphocytes activated?", and "Are cells in the TME contributing to immunotherapy resistance?".

2. **Optimizing sample processing and assay conditions so they are robust and reproducible**

   Because spatial signature development is a relatively new methodology, limited guidelines are available around sample collection, sample fixation, processing, and storage, and are areas where standardization is needed. Histopathology variables that can affect brightfield or monoplex immunofluorescence staining can also affect mIF results including tissue fixation parameters, tissue/blockage, and other pre-analytical steps.

3. **Intra- and inter-observer variability**

   Subtle variations in the reported expression level or staining patterns, and image classification parameters (such as section thickness and thresholds for feature segmentation) can influence biomarker use.

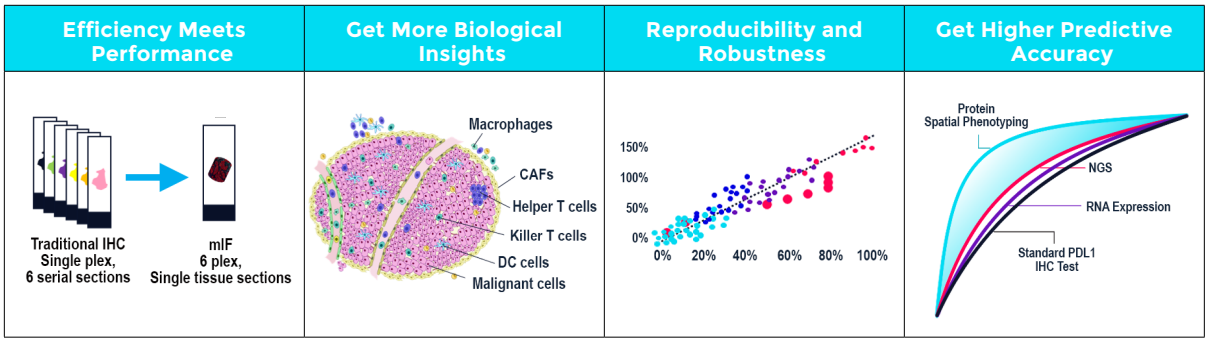

FIGURE 1. Spatial signatures can meet core requirements for a translational research platform. This process first starts with a combination of biological need and workflow efficiency. Then, before a platform can be implemented in clinical research, it is critical to establish the reproducibility and quality of the data. The final step in biomarker development is evaluating its predictive performance.

Examples of Predictive Spatial Signatures

Examining studies where spatial signatures were successfully used as biomarkers for immunotherapy can reveal common themes and important markers for TME characterization. The three examples below illustrate the potential of spatial signatures as clinical tools across a wide range of therapeutic areas.

SPATIAL SIGNATURE EXAMPLE #1:
A "Suppression Index" Marker for Oral Squamous Cell Cancer Prognosis

In one example where a well-designed spatial signature biomarker was developed and deployed, Feng Z, et. al. first observed that immune cell densities determined by single-plex IHC were insufficient markers to stratify subjects with oral squamous cell cancer (OSCC) [7]. Noticing that an increased number of regulatory T cells (FoxP3+ Treg) around cytotoxic T cell (CD8+) infiltrates correlated with lower overall survival, the authors hypothesized that the Treg cells could be suppressing the anti-tumor activity of the cytotoxic T cells. Consequently, they developed a "Suppression Index". This index reflected the number of FoxP3+ and PD-L1+ macrophage and tumor cells within a "3-lymphocyte" or 30µm distance around CD8+ T cells (FIGURE 2).

This index proved to be more predictive of overall survival than single-plex biomarkers. Patients who were ranked in the top 50% for both PD-L1+ and FoxP3+ cells had a high suppression index with a low overall survival while those that did not rank in the top 50% for either PD-L1+ and FoxP3+ cells had a low suppression index and a high overall survival rate. While these results indicate a favorable association between high CD8+ T cell density and OSCC patient survival, they demonstrate that the assessment of multiple parameters such as the suppression index, one example of a spatial signature, is a much stronger prognostic marker for subjects with OSCC [7].

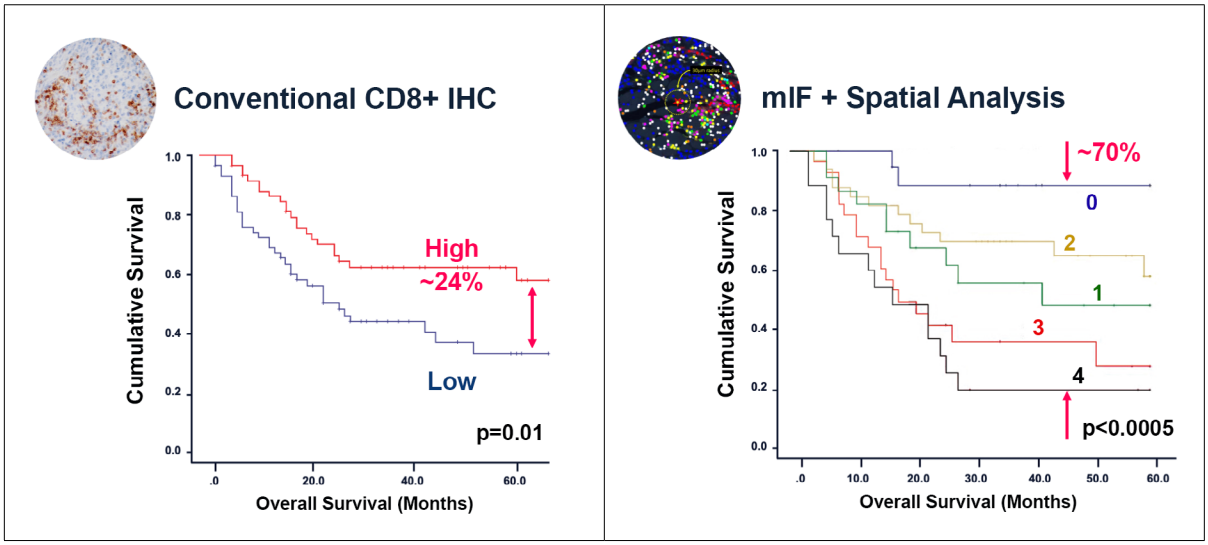

FIGURE 2. The spatial suppression index based on organization of tumor-infiltrating immune cells is a strong prognostic marker. Kaplan-Meier curve showing analysis of the entire cohort demonstrates the highly significant stepwise reduction of OS based on an increasing cumulative suppressive index, with 0 representing the lowest and 4 representing the most suppression relative to CD8+ T cells (right side) versus OS based on single marker CD8+ T Cell densities (left side) [used with permission; Ref 7].

mIF plus spatial analysis of the proximity of FoxP3+ and PD-L1+ to CD8+ cells led to a highly significant and granular stratification of overall survival (OS) based on an increasing Suppression Index and the development of a highly indicative prognostic marker (right panel). The prognostic value of this spatial signature was superior to the prognostic index of the single markers (FIGURE 2, left panel).

SPATIAL SIGNATURE EXAMPLE #2:

Mature Tertiary Lymphocyte Structures May Predict Response to Cancer Immunotherapy

Tertiary lymphoid structures (TLS) are lymph node-like, complex organizations of immune cells that can drive immune cell activation, potentially acting against tumors. Although several previous publications documented the correlation between TLS in the TME and immunotherapy response, the prognostic value of TLS had not been shown.

A multicenter study with a large sample size and an automatable workflow showed how developing a spatial signature based on TLS could be a promising, predictive biomarker for immune checkpoint immunotherapy in many kinds of solid tumors, including NSCLC, soft tissue sarcoma, bladder cancer, colorectal cancer, head/neck carcinoma, and renal carcinoma.

A retrospective analysis was performed on 540 tumors from patients (discovery cohort n=328, validation cohorts=212) before and after receiving immune checkpoint inhibitor therapy. Multiplex immunofluorescence imaging using Akoya Biosciences's PhenoImager® HT platform* plus quantitative analysis using inForm® software assessed TLS formation and maturity. Spatial phenotyping at scale revealed that mature TLS in tumor samples was predictive of a positive outcome in cancer patients treated with immune-checkpoint inhibitors (FIGURE 3).

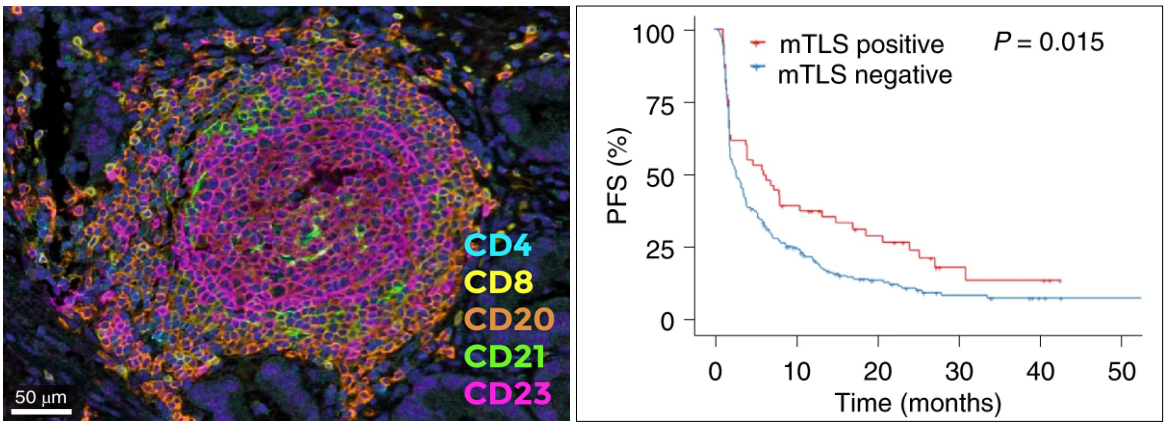

FIGURE 3. Mature TLS are predictive of increased survival in cancer patients treated with PD-1 or PD-L1 inhibitors. Primary pancreatic adenocarcinoma associated with CD23+ mature TLS, analyzed using mIF (left). Kaplan-Meier curves showing progression-free survival in the discovery cohort (328 patients) treated with anti-PD1/PD-L1 antagonist according to the mature TLS status (right; 6.1 months vs 2.7 months, p=0.015) [used with permission, Ref 8].

SPATIAL SIGNATURE EXAMPLE #3:

Novel Approach to Spatial Signature Development Provides General Framework for Developing Predictive Spatial Signature Biomarkers with Rigorous Clinical Validation

Melanoma researchers at Johns Hopkins University sought to develop spatial signatures of PD-1 therapeutic response using a discovery cohort of 53 subjects and a validation cohort of 45 subjects (samples were obtained from a different academic institution) with metastatic melanoma [6].

AstroPath™ is a sky-mapping algorithm that was developed at Johns Hopkins University to stitch together millions of images of billions of celestial objects, each expressing distinct signatures. Using a novel approach that applied the AstroPath algorithm and the PhenoImager platform* to mIF quantitative image analysis, John Hopkins researchers were able to develop spatial signatures from tumor tissues.

Using a six-plex (PD-1, PDL1, CD8, FoxP3, CD163, and Sox10/S100) Akoya mIF panel*, the researchers were able to develop 41 combinations of expression patterns and map relatively rare cells. This multifactorial analysis was used to study 10 features for predicting objective response in melanoma patients after immune checkpoint–blocking therapies (FIGURE 4a). Using these features, the researchers were able to stratify patients into poor, intermediate, and good prognosis groups (FIGURE 4b). Samples from subjects with a poor prognosis were characterized by high densities of tumor cells and CD163+ cells that lack PD-L1 expression, irrespective of whether other immune cells were present.

The area under the curve (AUC) values were assessed for the 10 spatial features for both the discovery cohort and the validation cohort and showed high accuracy for predicting objective response (AUC of 0.92 and 0.88, FIGURE 4c). These groups were predictive of long-term outcomes, i.e., overall survival and progression-free survival (FIGURE 4d), demonstrating the validation of this highly predictive mIF biomarker assay for metastatic melanoma.

Because the AstroPath approach addressed the specific challenges around image acquisition, analysis, quality control, and data handling associated with testing large numbers of biological samples, this methodology can be generally adopted as a framework for discovery as well as clinical validation of spatial signatures.

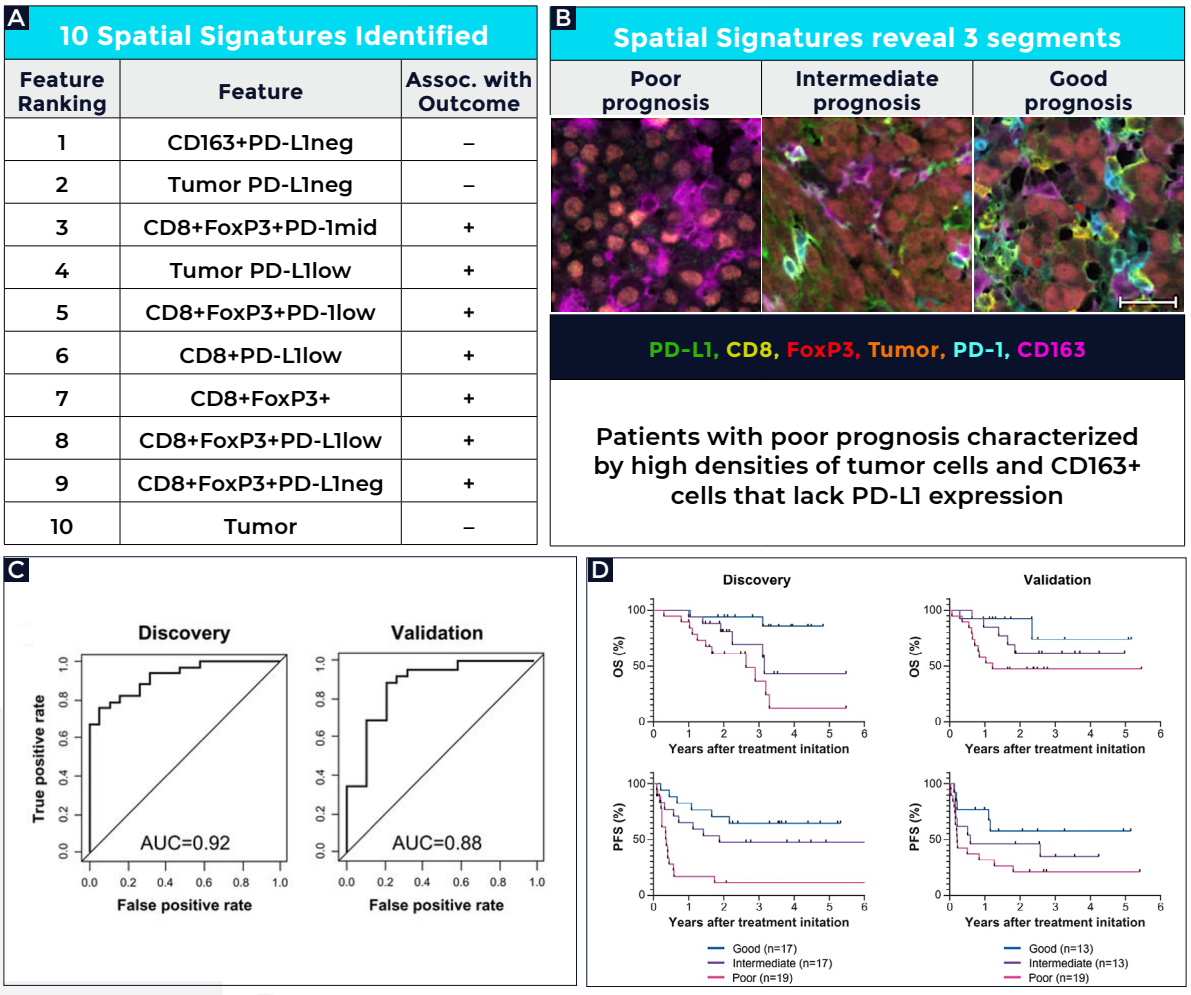

FIGURE 4. Developed using the AstroPath approach and the PhenoImager workflow, spatial signatures of melanoma revealed disease predicted response to PD-1 therapy [used with permission, Ref 6].

A Six-Plex mIF Workflow Balances Content and Throughput for Biomarker Discovery and Validation

As shown in the examples above, developing novel spatial biomarker signatures and establishing their potential clinical significance requires thoughtful panel design, studying large sample cohorts (tens to hundreds of samples) and a high throughput approach, using a workflow that can stain, image, and process whole slide imagery in tens of minutes each, versus hours or days. An ideal spatial signature biomarker may ultimately inform real-time clinical decision-making.

Using preconfigured multiplex panels can be an efficient way to quickly assess the prognostic value of multiple markers on tissues of interest. The number of markers within a multiplex panel affects the efficiency with which staining, and analysis can be optimized. To this end, six markers are frequently used as a compromise between significance and ease of panel optimization [6].

Typically, a six marker panel should include the following components:

- **1 marker** for labeling the tumor in contrast with the associated stromal microenvironment
- **2 or 3 markers** for key biological mechanisms that provide spatial landmarks to guide image analysis to support accurate phenotyping
- **2 to 3 markers** that provide additional phenotyping for the specific cancer type and when combined with the above provide a robust and reproducible spatial signature

Fewer than six markers can leave out critical information important to robust signatures, while greater than six forces the use of technologies that are an order of magnitude slower than needed for translational studies and trials and are not well suited to translation into the clinic.

Current whole-slide, single-cell imaging methods for higher-plex assays (more than six marker panels) are powerful; however, the relatively higher cost and lower throughput of higher-plex panels pose barriers to adoption in pre-clinical and clinical research workflows. Costs can add up to over $1,000 per slide, and throughput can be as low as one slide per day.

Establishing Validation, Scaleup, and Reproducibility

Once a panel's marker composition has been determined, a high throughput assay detection platform is indispensable for scaling up signature validation and eventual implementation in clinical research. A high throughput platform that is sufficiently rapid and accurate enables users to study dozens to hundreds of samples each week, depending on throughput requirements.

Before a platform can be implemented in the clinic, it is important to establish the reproducibility and quality of the data. The platform needs to be analytically validated, particularly in comparison to current gold-standard methods.

In the first multisite study of a spatial phenotyping platform, 4 academic medical centers, one pharmaceutical company and Akoya Biosciences assessed the inter- and intra-site reproducibility of a 6-plex mIF assay [9]. The results of the study showed that the PhenoImager® HT workflow* consisting of Opal™ staining on the Leica® Bond™ RX

autostainer, imaging with the PhenoImager HT and analysis with Akoya's inForm software is the first spatial biology platform to demonstrate reproducibility suitable for clinical research applications (FIGURE 5).

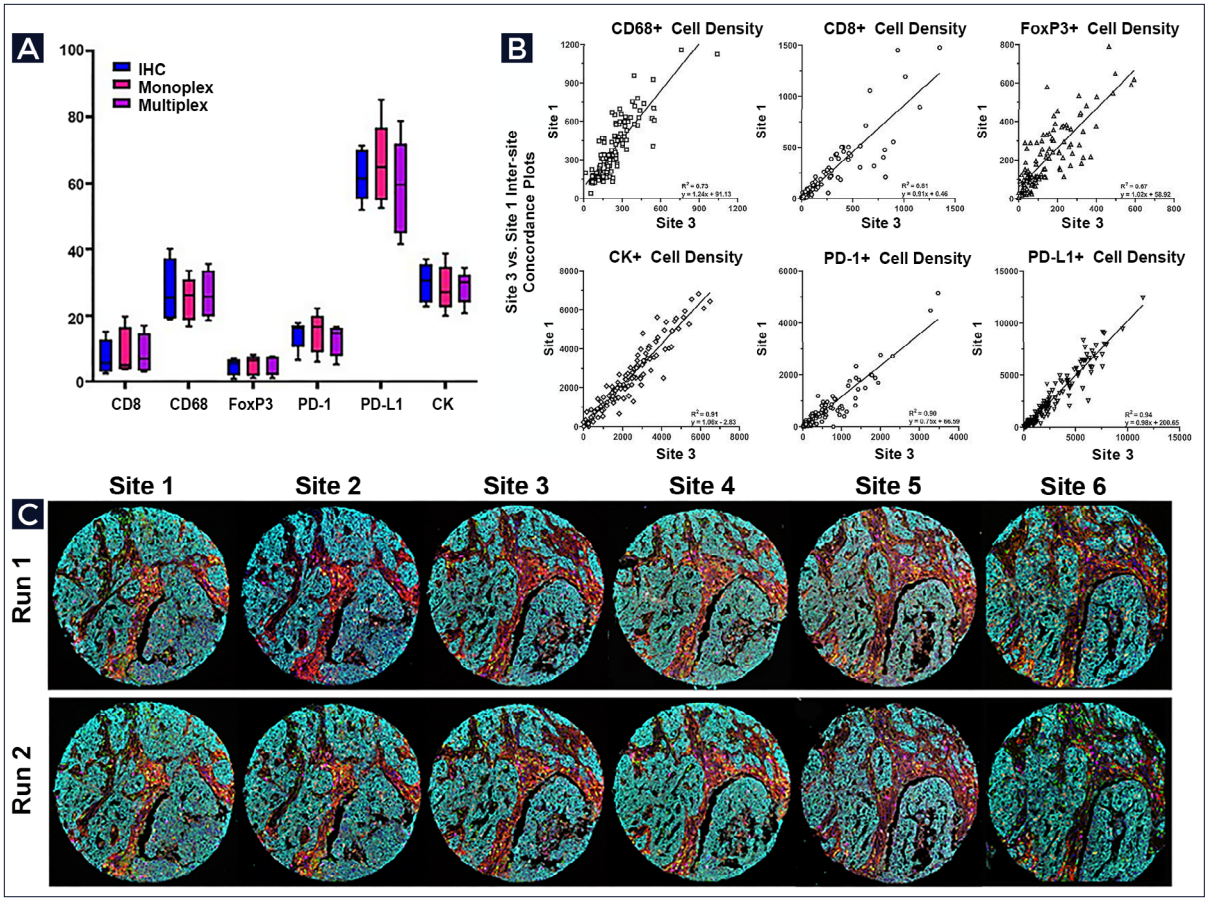

FIGURE 5. Multi-site study validates the inter- intra-site reproducibility of a 6-plex mIF assay platform. (A) Quantitative comparison of percentage of cells phenotyped as 'positive' for each marker by staining approach (chromogenic IHC, monoplex IF, and multiplex IF). Plot shows median and IQR, with whiskers showing min to max for each marker. (B) Representative inter-site cell density concordance plots for each marker, CD68, CD8, FOXP3, PD-1, PD-L1, and CK (tumor cells). (C) Strong inter- and intra-site concordance was observed for the cell lineages markers assessed in breast carcinoma TMA [9; image courtesy of Bethany Remeniuk, Akoya Biosciences].

Researchers first tested both the monoplex IF and multiplex IF stains for equivalence to chromogenic IHC. Comparing the percentages of positive cells for each marker by chromogenic IHC, monoplex IF, and multiplex IF staining approaches showed equivalence across all three staining modalities (FIGURE 5a). Cell densities as assessed by the different cell lineages markers showed excellent concordance across sites (FIGURE 5b). Strong intra- and inter-site concordance was observed for staining reproducibility using a breast carcinoma tissue microarray (TMA) (FIGURE 5c).

In addition to staining reproducibility and cell densities, excellent inter-site concordance of % PD-L1 expression by cell type and PD-1/PD-L1 proximity analysis was observed, demonstrating the reproducibility, analytic performance, and robustness of Akoya's PhenoImager HT platform* for spatial phenotyping and the development of predictive biomarkers for preselecting responders for immunotherapy. Akoya Biosciences has also received ISO 13485:2016 certification for its quality management system [10].

Clinical validation is ultimately the most important step in developing spatial signature biomarkers that are useful for clinical research. Clinical validation requires showing that a spatial signature assay's AUC can be replicated in an independent cohort of samples from a different institution than the discovery cohort that was used to train the algorithm originally used to develop the signature (performed in studies described in examples #2 and #3 above and shown in FIGURE 4c).

The Right Tools Accelerate Assay Optimization of Spatial Signature Biomarkers

The fastest path to developing spatial signature biomarkers requires a solution that easily integrates staining, imaging, and analysis using existing workflows while providing accuracy and reproducibility.

The PhenoImager workflow* from Akoya has consistently demonstrated robustness in each of these aspects, because it combines the best, fit-for-purpose **reagents, instruments, and analysis tools.**

The Right Reagents—Opal-TSA Reagents* and PhenoCode Signature Panels*

Opal-TSA Reagents: The adoption of tyramide signal amplification with Opal dyes (mIF Opal-TSA) is enabling researchers to apply mIF to clinical research workflows. The Opal-TSA method involves the use of standard, unlabeled primary antibodies, a secondary antibody HRP, and Opal-TSA fluorophores that are used for detection by covalently labeling the epitope. After labeling, antibodies are removed in a manner that does not disrupt the Opal fluorescence signal.

Opal-TSA Reagents have enabled researchers to:

- Use the best primary antibodies regardless of species, without cross-reactivity
- Improve sensitivity, dynamic range, and resolution
- Achieve better correlation between protein expression and signal intensity
- Increase plexing for multiple biomarker detection

Given the ever-growing body of published Opal-TSA protocols, researchers can refer to best practices for establishing reproducible and scalable workflows, encompassing antibody panel development, staining, image acquisition, and analysis [11].

PhenoCode Signature panels, a suite of pre-configured immunofluorescence panels, allow for the rapid and systematic analysis of the tumor microenvironment with minimal assay development and optimization (FIGURE 6). These panels offer four key advantages to support spatial signature development:

- **Relevant Content**—Designed to be complementary to one another, the panels ask the most common questions related to the presence, distribution, subtype and status or functional state of tumor and immune cells in the TME.

- **Flexible**—Each PhenoCode™ Signature 5-plex panel allows for the easy integration of one additional marker, enabling analysis of additional cell phenotypes or specific research questions.
- **Fast**—Out-of-the-box solutions are optimized and validated to enable the rapid development of spatial signatures by shortening the assay development time by 3X compared to custom-built 6-plex panels.
- **Scalable**—Optimized for integration with the PhenoImager workflow, the panels are designed for automation and high throughput.

PhenoCode Signature 6-plex Panels (5-plex base + 1 custom marker)

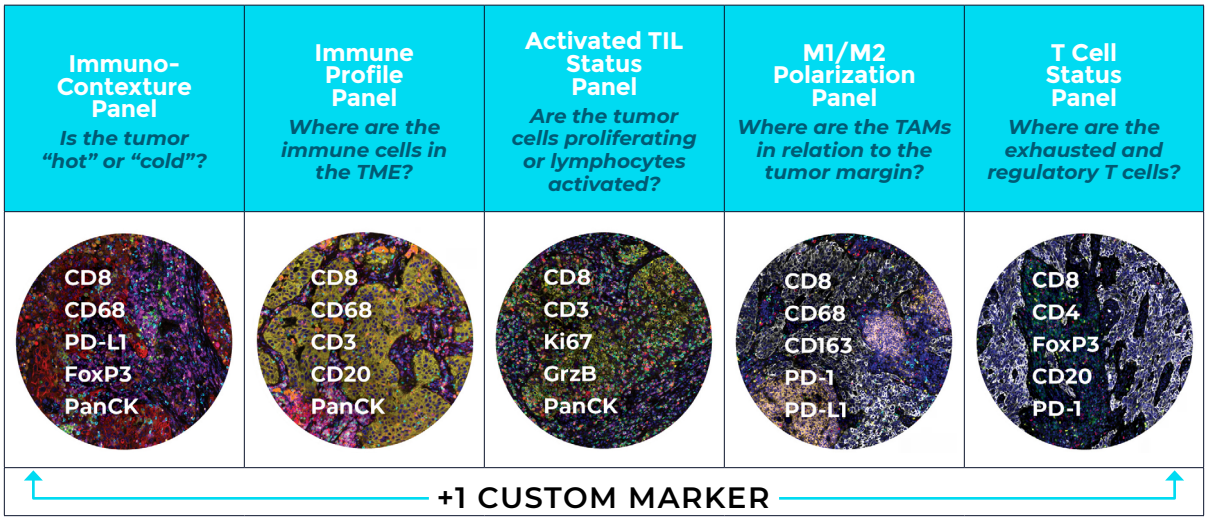

FIGURE 6. PhenoCode Signature Panels enable interrogation of the TME characteristics that often determine response to combination immunotherapies.

The Right Instruments: PhenoImager HT and PhenoImager Fusion Systems*

Leveraging patented multispectral imaging (MSI) technology, the PhenoImager solution provides instruments that accurately quantify multiple immune phenotypes while capturing their tissue context and spatial distribution within the TME. This powerful but easy-to-use digital pathology platform provides rapid, whole-slide image acquisition (6-plex whole-slide scan in under 20 minutes) with walk-away automation.

The Right Analysis Solution

The PhenoImager platform* generates large volumes of whole-slide MSI data, which can be challenging to analyze at scale. Fortunately, Akoya's suite of image analysis software solutions (InForm tissue analysis software, phenoptrReports package, and the Phenochart application) combine the latest technologies with algorithms and intuitive, easy-to-use interfaces, enabling scientists to visualize, analyze, quantify and phenotype cells labeled with multiple biomarkers in situ in FFPE tissue sections.

Akoya's technology partnerships, third-party solutions, and open-source applications provide users with additional options for interrogating and visualizing spatial phenotyping data.

CONCLUSION

Spatial Signatures Poised to Enter the Clinic

Ultimately, successful biomarker development is determined by evaluating the biomarker's predictive performance, and translational researchers are quickly unlocking the potential of spatial phenotyping. A rapidly growing number of publications are showing that spatial interactions, locations, and distances between tumor cells and immune cells can predict response [12, 13].

While challenges remain, recent advances provide evidence that the combination of PhenoCode Signature Panels, PhenoImager HT system, and associated software applications represent a promising new workflow for developing spatial signature biomarkers that are more predictive than the current standard-of-care.

A rapidly growing number of publications are showing that spatial interactions, locations, and distances between tumor cells and immune cells can predict response.

Akoya and its collaborators are developing companion diagnostics for targeted therapeutics based on spatial signatures. In a second collaboration, Akoya is developing a staining workflow optimized to support the application of spatial biology in clinical and diagnostic research. Finally, rapidly advancing image analysis methods, such as those using unsupervised graph learning and artificial intelligence-powered phenotyping, are showing how mIF and protein spatial phenotyping can improve stratification and clinical outcomes [14].

Learn more at **www.akoyabio.com/phenocode-signature/**

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