PhenoCode Signature Panels: Decoding the Science of Response to Cancer Therapy

HIGHLIGHTS:

- Spatial signatures are a promising class of biomarkers for predicting response to cancer immunotherapy.
- Developing spatial signatures as clinically useful biomarkers requires efficient marker selection and rapid workflows to speed up assay development.
- PhenoCode™ Signature Panels, together with the PhenoImager™ HT, provide a scalable, rapid, and flexible workflow for quantifying spatial biomarkers using multiplex immunohistochemistry (IHC).
- Deploying the workflow on 37 pretreatment biopsies from non-small cell lung cancer (NSCLC) cases revealed multiple spatial signatures that were correlated to treatment outcome.

INTRODUCTION

The Promise of Spatial Signatures: Better Patient Stratification

Cancer immunotherapies utilizing immune checkpoint inhibitors (ICIs) have led to improved patient outcomes, but patient stratification remains a challenge. In the search for clinically useful biomarkers to select responders, spatial biology approaches are crucial, as these methods address all aspects of the interaction between the tumor and the tumor microenvironment (TME). A 2019 meta-analysis (**Lu, Stein, et al., 2019**) has shown that spatial phenotyping, enabled by multiplex IHC (brighfield and immunofluorescence), more accurately predicts patient response to anti-PD-1/PD-L1 immunotherapy than other biomarker modalities.

Building on this seminal meta-analysis, spatial biology signatures of immunotherapy response are emerging as a biomarker class with high predictive accuracy.

Developing verified multiplex immunofluorescence (mIF) panels, however, has been challenging because of the multiple factors affecting assay optimization. Imaging protocols for multiplex assays are affected by relative epitope stability, optimal antibody concentrations, antibody staining order, fluorophore-antibody pairings, and denaturing protocols. Assay development is further complicated by factors affecting image acquisition and analysis, such as co-expressing biomarkers, fluorescence crosstalk, and relative fluorophore intensity (**Pulsawatdi VA, Craig S, et al; 2020**).

Benefits of PhenoCode Signature Panels: Shortening the Path to Prediction

PhenoCode Signature Panels address the challenges of developing spatial signatures and mIF assays, speeding up signature development by three-fold compared to do-it-yourself (DIY) panels.

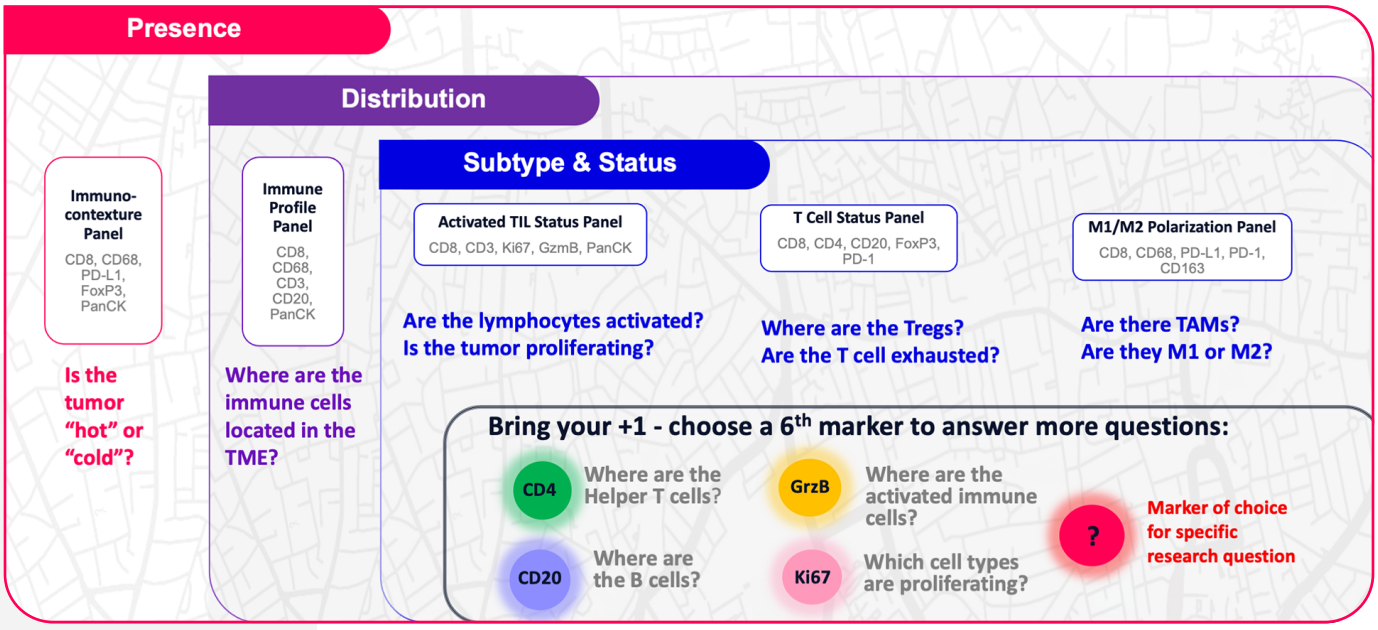

FIGURE 1. Asking the right questions, with the right biomarker content and the flexibility to add a marker of choice, enables systematic analysis of tumor immune response. The five PhenoCode Signature Panels are designed to be complementary and have the relevant content to address key questions shown in this figure to characterize the TME. The panels also allow for the addition of a sixth marker, which can be selected from the list of à la carte antibodies or a marker of choice. This allows the mapping of additional phenotypes and answers additional questions.

This accelerated workflow is enabled by:

1. RELEVANT: Customer feedback, combined with expert curation of hundreds of publications, informed the design of a series of complementary panels to ask fundamental questions related to the presence, distribution, subtype and functional state of tumor and immune cells (FIGURE 1). Each panel included the most relevant biomarkers to answer a key question about the TME.

2. FLEXIBLE: An additional marker of choice can be added to each PhenoCode Panel so users can answer specific and/or novel questions.

3. FAST: By providing base panels of five markers that have been optimized for the PhenoImager workflow, PhenoCode Signature Panels accelerate the development of 6-plex assays by at least three-fold (based on internal data and data from three early-access customer sites) and lowers the barrier of expertise needed to develop multiplex assays. This fast, out-of-the-box solution offers many advantages, particularly for projects that have short delivery timelines.

4. SCALABLE: PhenoCode Signature Panels can be integrated into a workflow amenable to automation and higher throughput. Whole slides can be stained with these 6-plex panels using the Leica® Bond RX™ autostainer (~12.5 hours, 30 slides/run) and imaged using the PhenoImager HT system (less than 20 minutes/slide). This high throughput enables the analysis of sufficiently large numbers of samples to yield statistically significant data for developing predictive biomarkers.

PHENOCODE SIGNATURE PANEL DEVELOPMENT AND TESTING

Workflow Efficiency With Gold-Standard Performance

To verify the PhenoCode Signature Panels, mIF staining was tested for equivalence to chromogenic IHC (FIGURE 2). Representative images for each marker showed comparable staining patterns to chromogenic IHC (as scored by a pathologist) on lung cancer serial sections, indicating that the detection of the markers in each panel was accurate. Additionally, marker intensity in the multiplex assay were determined to be within 20% CV (coefficient of variation) when compared with the equivalent monoplex staining intensity, indicating minimal or no "umbrella" effect [for explanation see **Hoyt CC 2021**].

Reproducibility with Integration of Different Markers

To test the reproducibility of the PhenoCode Signature Panels, two unique 6-plex panels were created from a 5-plex base panel. Staining of the markers was compared across three serial tonsil and lung cancer tissue sections (technical replicates) and three different tonsil and lung cancer tissues (biological replicates). Results showed that the data were not affected by swapping in a different sixth marker (FIGURE 3). Within the context of the two different PhenoCode Immuno-Contexture Panels, CD8, CD68, and FoxP3 stains all detected similar cell densities between responder (R) vs. non-responder (NR) lung cancer tissues.

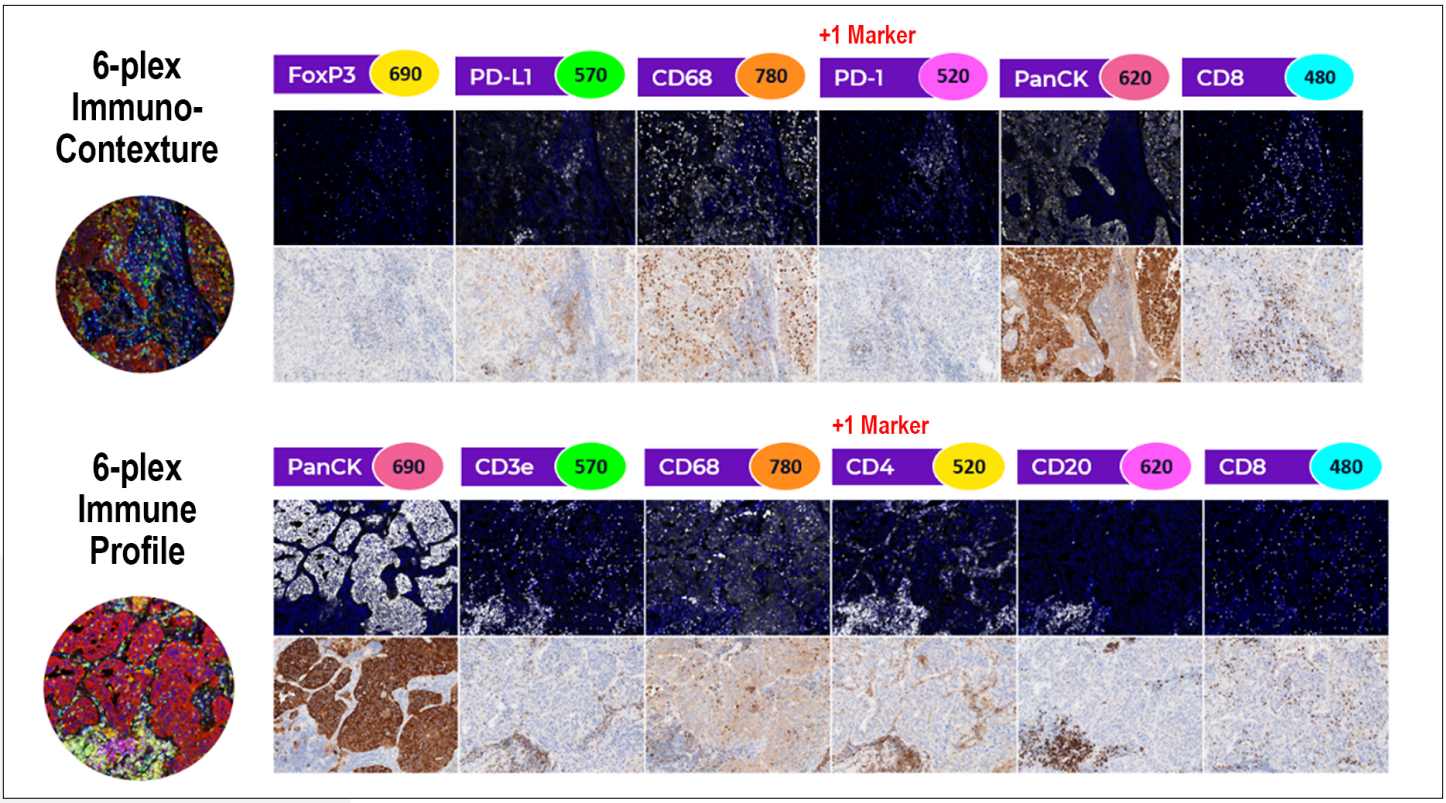

FIGURE 2. Comparable staining patterns generated using multiplex IF PhenoCode Signature Panels and chromogenic IHC staining. Benchmarking of two panels is shown: a 6-plex PhenoCode Signature Immuno-Contexture Panel (top), where the PD-1 antibody was paired with Opal 520 and added to the 5-plex base panel, and a 6-plex PhenoCode Signature Immune Profile Panel (bottom), where the CD4 antibody was paired with Opal 520 and added to the 5-plex base panel. (Image courtesy of Jacob Circelli, Akoya Biosciences)

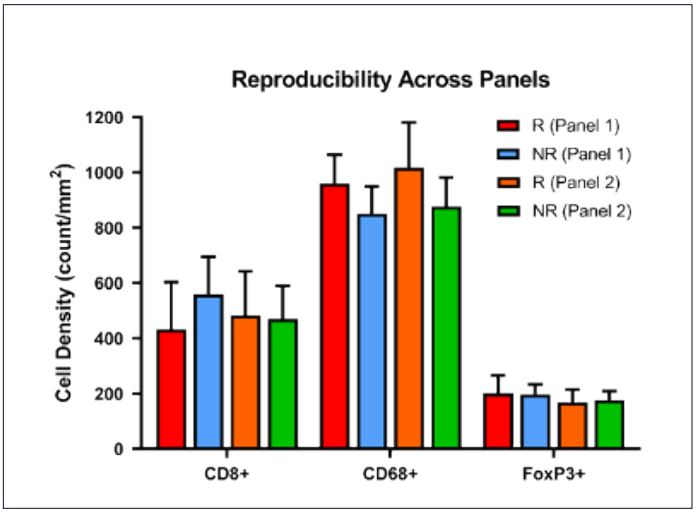

FIGURE 3. Intra-panel reproducibility is not impacted by swapping open channel markers. Three tissue microarray (TMA) slides comprising 41 formalin-fixed paraffin-embedded (FFPE) NSCLC patient samples from second-line PD-L1/PD-1 ICI-treated cohorts (16 responders and 25 non-responders) were stained using PhenoCode Signature Immuno-Contexture Panels with two different markers, PD-1 (Panel 1) and CD20 (Panel 2) on a Leica Bond RX™ autostainer. Data also shows no significant differences from single marker analysis in responder/non-responder (R/NR) cohorts. (Image courtesy of Bethany Remeniuk, Akoya Biosciences)

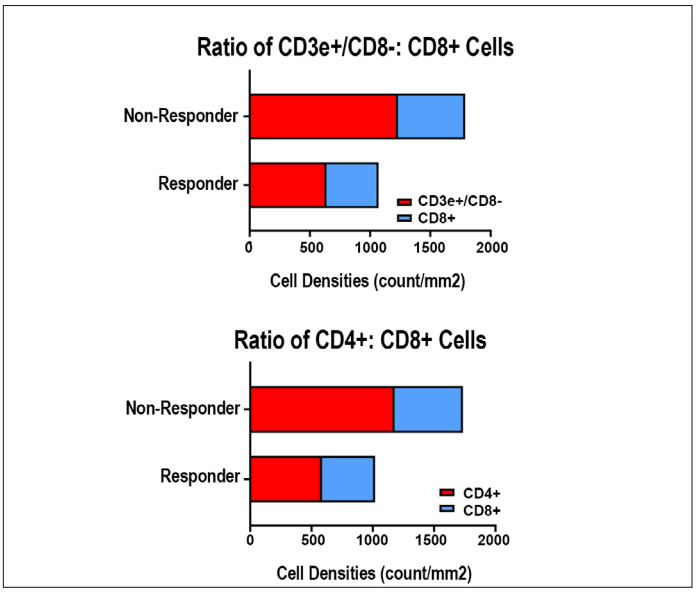

FIGURE 4. Conjugated antibodies are specific and provide accurate staining. Tissue microarray (TMA) slides for the cohort (see Figure 3) were stained using PhenoCode Signature Panels. Cell density of CD4+ T cells were measured using two different markers: quantifying CD3e+/CD8- T cells (left) and quantifying CD4+ T cells (right). CD8 was also measured in each experiment. The cell density plots show similar cell densities for CD4+ cells and for CD8+ cells when calculated using different phenotyping markers. (Image courtesy of Bethany Remeniuk, Akoya Biosciences)

Barcoded Antibodies Offer Specificity With Flexibility

PhenoCode Signature Panels are powered by a novel, barcoded antibody chemistry. Barcoding enables easy panel design, while amplification provides gold-standard sensitivity and accuracy. PhenoCode Signature Panels stained on Leica BOND RX and scanned on PhenoImager HT generated comparable intensities when looking at serial sections with CVs less than 20%. The signal to background ratios were determined to be a minimum of 20:1 for all markers, with individual marker staining qualitatively evaluated to be specific to the respective Opal, with no visible crosstalk in adjacent Opal channels. Assay sensitivity was also examined by looking at low expressors for both PD-1 and PD-L1.

Whenever antibodies are conjugated, however, assay developers must assess whether the conjugation chemistry interferes with the specificity and accuracy of staining. Quantifying a cell population using two different sets of markers showed that the calculated cell density remained constant across the different staining methods, confirming that PhenoCode Panel antibodies were specific, accurate, and unaffected by the conjugation chemistry (FIGURE 4).

Gentle, Fast, Sensitive: Advantages of PhenoCode Staining

PhenoCode Signature Panels are used according to an optimized 7-step workflow (FIGURE 5).

Key advantages of multiplexed detection using PhenoCode chemistry:

- Gentler conditions (de-hybridization being gentler than antibody stripping) preserves tissues and enables easier panel optimization.
- Antibody cocktailing — more efficient de-hybridization.
- No need to optimize a secondary antibody (lower background, higher specificity).
- Utilization of Opal-TSA signal amplification for high sensitivity.

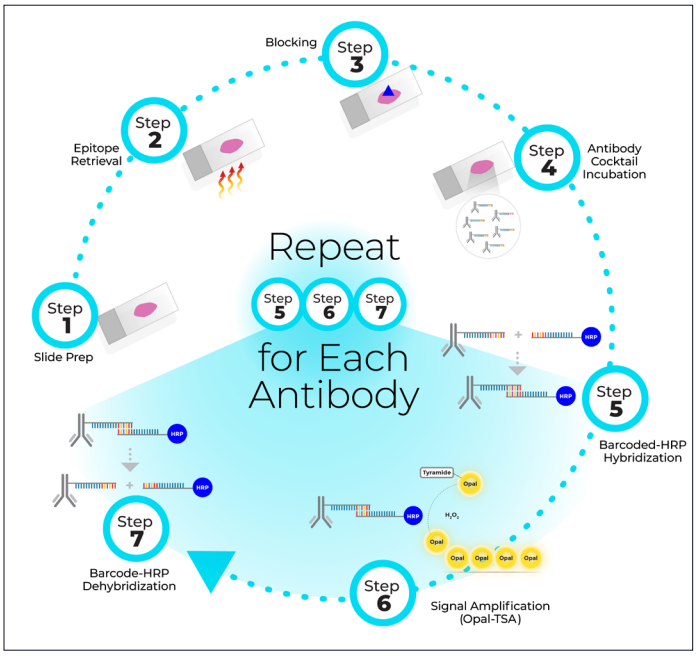

FIGURE 5. Seven-step staining workflow for PhenoCode Signature Panels. Following the traditional slide preparation steps of baking, dewaxing, and epitope retrieval and blocking, the slides are stained with the panel's primary antibody cocktail, in which the antibodies have been conjugated to unique oligo barcodes. After antibody incubation, a single antibody is revealed one at a time, beginning with the hybridization of a complementary oligo barcode conjugated to an HRP. Signal amplification is then performed using the Opal–TSA chemistry. Once complete, the HRP conjugated oligo is dehybridized. The process (steps 6–8) is repeated for each antibody.

FLEXIBILITY ALLOWS LAYERED INTERROGATION OF THE TME

Each 5-plex PhenoCode Signature Panel allows for the addition of a sixth marker, which can be selected from the list of à la carte antibodies or a marker of your choice. This allows investigators to map additional phenotypes and answer additional questions regarding tumor/microenvironment interactions, such as:

- Where are the Helper T cells?
- Are the T cells exhausted?
- Which cell types are proliferating?
- Where are the B cells?
- Where are the activated immune cells?
- ...and YOUR questions of interest!

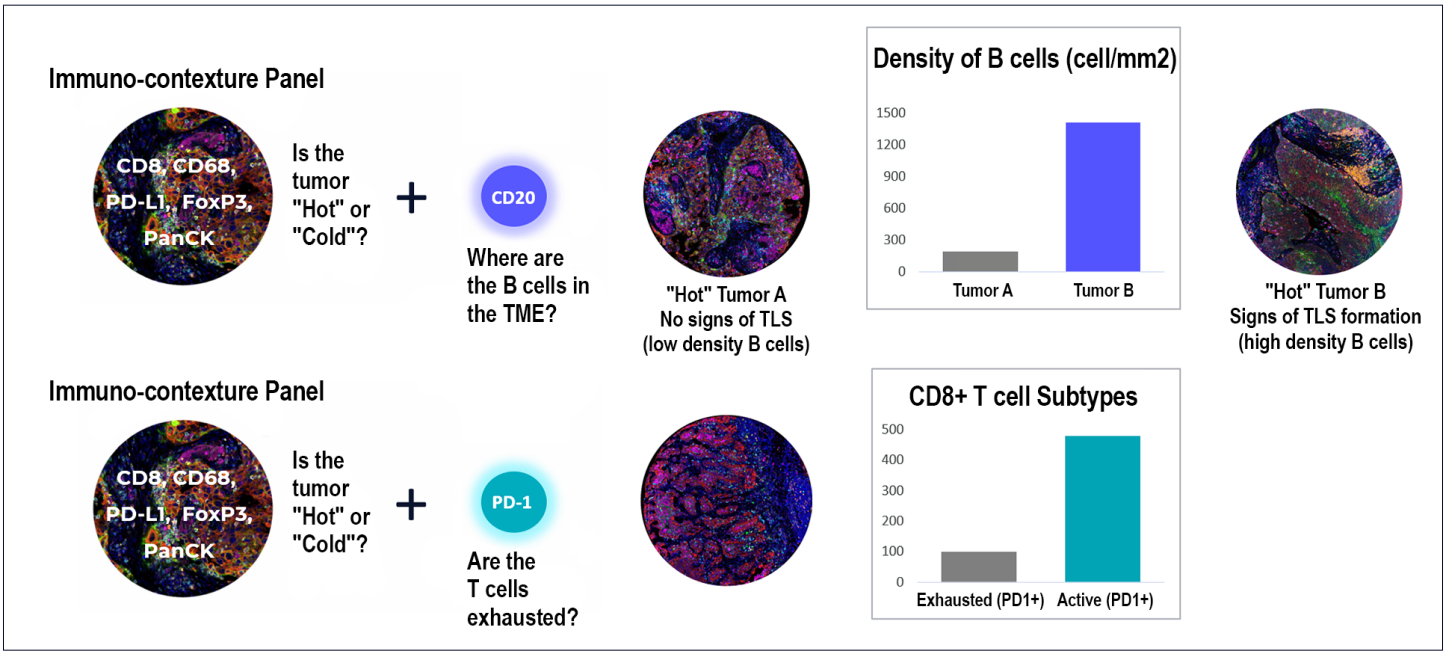

FIGURE 6. Answer layered questions; adding two different markers to the same panel enables assessment of different parameters. The PhenoCode Signature Immuno-Contexture Panel was first combined with CD20 to enable analysis of B cell density and associated features. When the panel was instead combined with PD-1, the ratio of CD8+PD1+ to CD8+PD1- T cells within the TME could be examined to determine T cell exhaustion. (Image courtesy of Bethany Remeniuk and Agnes Haggerty, Akoya Biosciences)

CASE STUDY

SINGLE-CELL SPATIAL PHENOTYPING OF PRE-IMMUNOTHERAPY NSCLC BIOPSIES: PhenoCode Signature Panels Were Used to Develop a Spatial Signature

Among lung cancer cases, 85% are non-small cell lung cancer. Of the NSCLC patients who receive immune checkpoint inhibitor (ICI) immunotherapy, only about 15%–20% respond. In this retrospective study, 21 pretreatment biopsies from NSCLC patients treated with an anti-PD1 therapy were phenotyped and used to develop a spatial signature of ICI response (FIGURE 7).

The first step in developing a spatial signature involved ultrahigh-plex spatial phenotyping, which revealed distinct immune profiles between complete responder tumors and stable disease tumors (**Sadeghirad, H., Liu, et. al., 2023**). However, while there were clear differences in the immune cell makeup (for example, B cells), it was difficult to establish statistical significance due to the low throughput of the analysis (FIGURE 8).

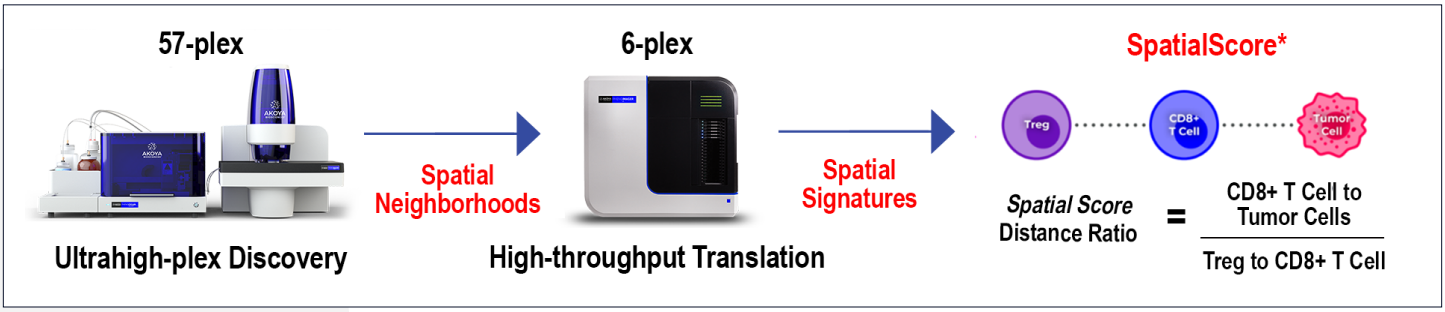

FIGURE 7. Discovery to translational workflow. NSCLC pretreatment core biopsies (FFPE) were phenotyped using a 57-plex antibody panel on PhenoCycler-Fusion using markers for cell lineage, immune activation, checkpoints, cellular energetics, and more. Serial sections were then stained with PhenoCode Signature Panels: Immune Profile Panel + CD4 in the open channel (PSP-IP) and Immuno-Contexture Panel + PD-1 in the open channel (PSP-IC); these samples were imaged on the PhenoImager HT system with preoptimized acquisition parameters and analyzed with the inForm® application. Spatial analyses, including the SpatialScore vs. ICI responses, were implemented in a custom analysis pipeline (Philips 2011) and used to differentiate responders from non-responders. (Image courtesy of Ning Ma, Akoya Biosciences and Dr. Arutha Kulasinghe, University of Queensland)

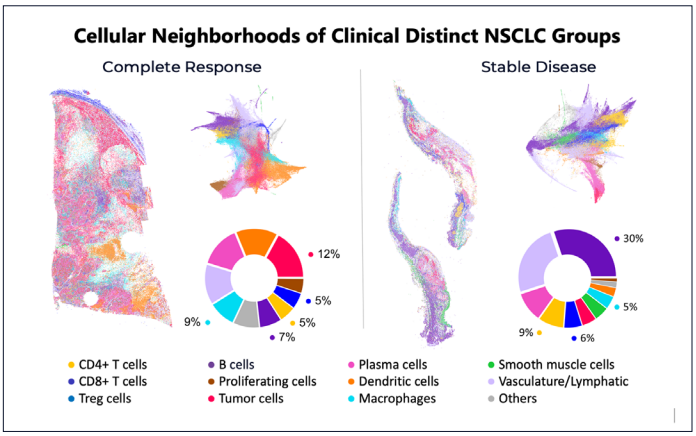

FIGURE 8. Ultrahigh-plex phenotyping of complete responder tumors vs. stable disease tumors. NSCLC pretreatment biopsies from the complete response group and the stable disease group were phenotyped; 298,084 cells and 254,272 cells were phenotyped, respectively, and 11 cell phenotypes identified with proportions shown in the pie charts. (Image courtesy of Ning Ma, Akoya Biosciences and Dr. Arutha Kulasinghe, University of Queensland)

Next, two different 6-plex PhenoCode Signature Panels were used to perform spatial phenotyping on the same tissues as used in the ultrahigh-plex phenotyping step (FIGURE 9). The 6-plex spatial phenotyping validated the diverse NSCLC immune landscape observed with the 57-plex panel and verified no statistical difference in total number of phenotyped cells between the responder and non-responder groups.

Finally, cell neighborhood analyses were performed, which were able to quantify the distinct spatial biology between responder vs. non-responder tumor microenvironments (FIGURE 10). NSCLC biopsies were found to contain seven distinct cellular neighborhoods. Notably, tumor infiltrating macrophages and tumor cells showed fewer direct interactions in non-response groups.

The SpatialScore paradigm was applied to facilitate the evaluation of the prognostic value of spatial phenotypes characterized in this study. The SpatialScore was originally established as the ratio of the physical distance between CD4+ T cells and the nearest tumor cell, relative to its nearest Treg (T regulatory cell; **Philips et al., 2021**). This study expanded the SpatialScore parameter to also reflect the interactions between CD8+ T cells and macrophages and CD8+ T cells and Treg.

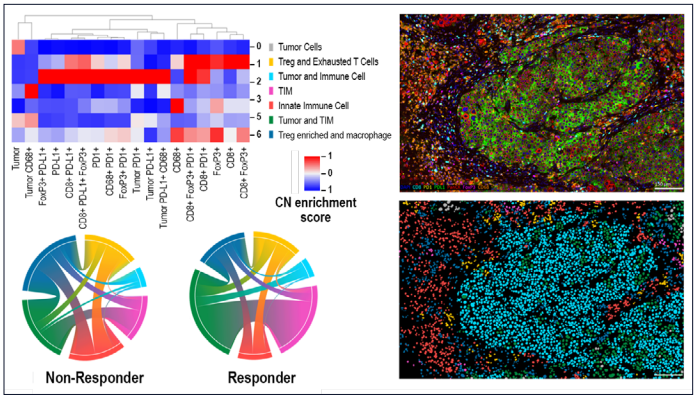

FIGURE 10. Cellular Neighborhood (CN) analysis of NSCLC tissues analyzed with the PhenoCode Signature Immuno-Contexture Panel (CD8, PD1, PD-L1, PanCK, FoxP3, CD68) showed differences in CN interactions between responders and non-responders. Shown here on the top right is a representative image of a pretreatment biopsy and below it, its CN representation. The heatmap on the top left summarizes the seven cellular neighborhoods. The Circos plots on the bottom left show dynamic nearest neighbor interactions between cellular neighborhoods across cohorts. (Image courtesy of Ning Ma, Akoya Biosciences and Dr. Arutha Kulasinghe, University of Queensland)

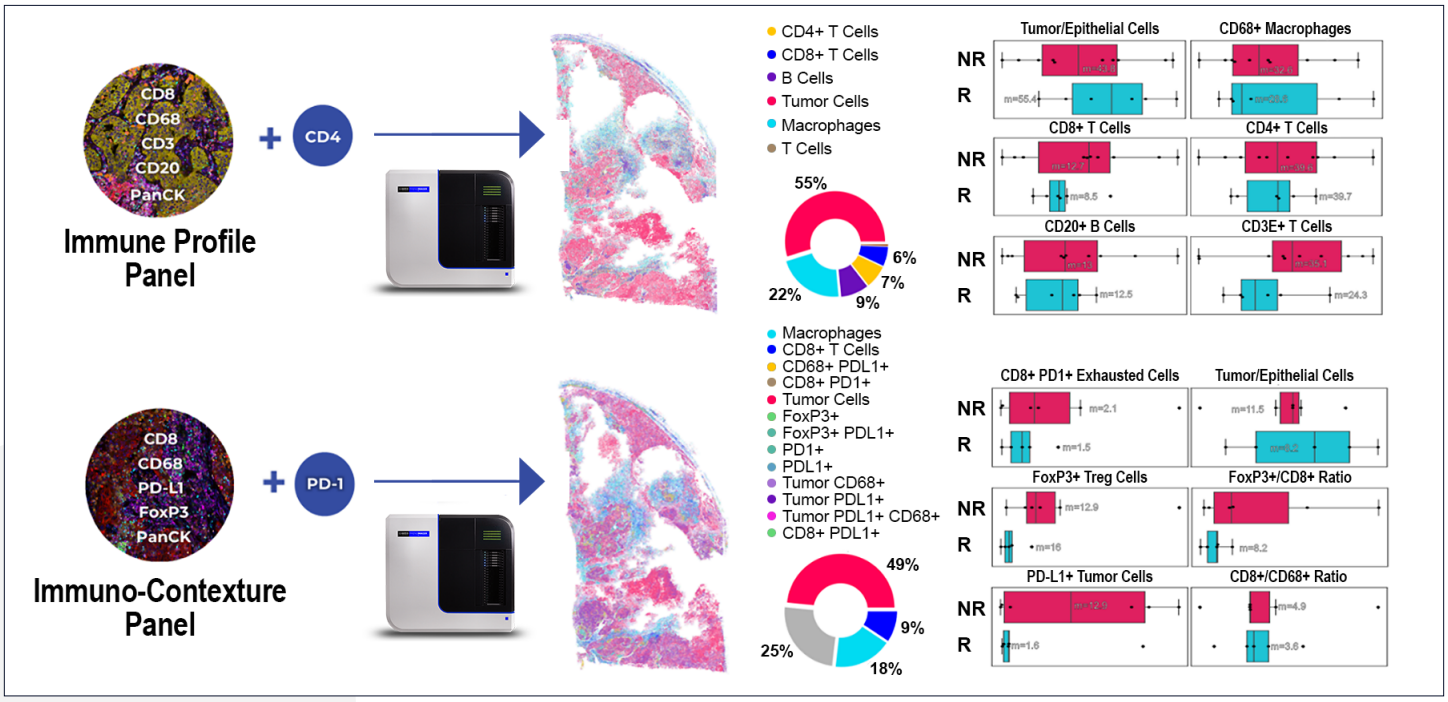

FIGURE 9. NSCLC pretreatment biopsies from responders and non-responders were stained with the Immune Profile Panel (top) and the Immuno-Contexture Panel (bottom). Cell quantification (m=median), including PanCK+ tumor/epithelial cells, CD68+ macrophages, CD20+ B cells, and various T cells, showed no significant difference across pooled pretreatment biopsies from responders vs. non-responders. (Image courtesy of Ning Ma, Akoya Biosciences and Dr. Arutha Kulasinghe, University of Queensland)

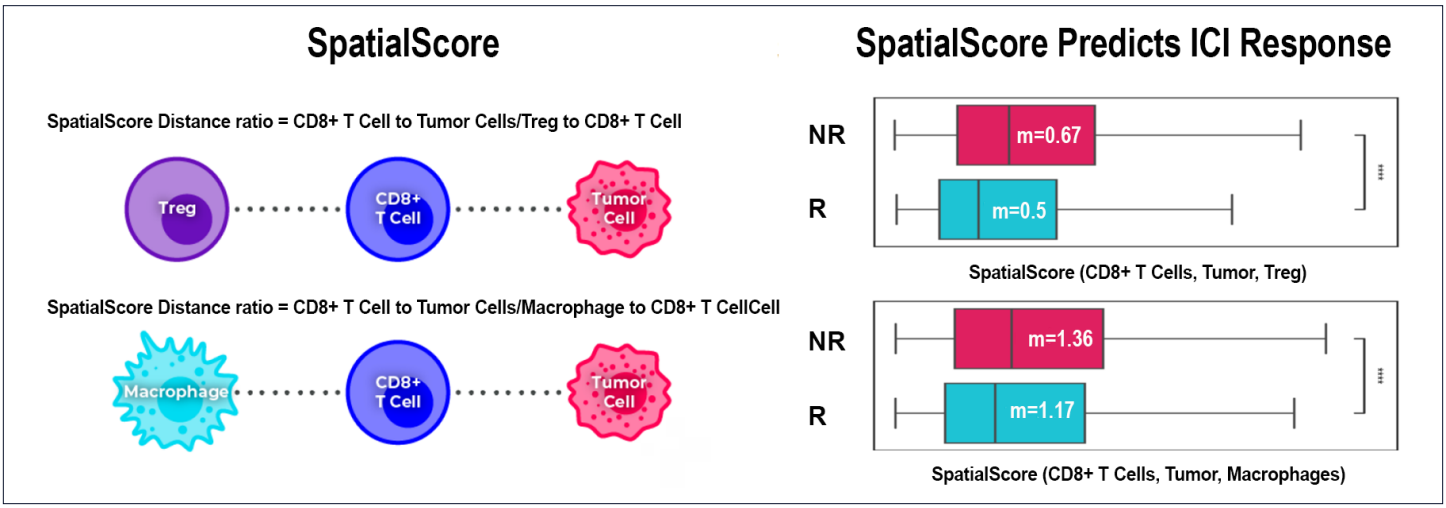

FIGURE 11. SpatialScore assessment, enabled by PhenoCycler Fusion, PhenoCode Signature Panels, and PhenoImager HT discovery to translational workflow, is a predictive spatial signature for treatment outcomes. (Image courtesy of Ning Ma, Akoya Biosciences and Dr. Arutha Kulasinghe, University of Queensland)

As shown in the left side of FIGURE 11, when CD8+ T cells are closer to macrophages or Treg than to tumor cells, a higher SpatialScore is noted. The pooled SpatialScores from pretreatment biopsies were significantly higher in the non-responder condition when compared to the samples from responders (right side of FIGURE 11). A high spatial score can thus be interpreted as higher CD8+ T cell suppression, lower anti-tumor activity, and lower survival rate.

This study illustrated a uniquely comprehensive single cell spatial phenotyping analysis of pretreatment NSCLC biopsies from a single-agent anti-PD-1 clinical trial. The data illustrated the diverse immune microenvironment of NSCLC but indicated that immune cell quantification was insufficient to stratify patient cohorts. The combination of Akoya's PhenoCycler Fusion and PhenoImager solutions allowed for the discovery of distinguishing features between responders and non-responders. Together with the throughput provided by PhenoCode Signature Panels, the results revealed the impact and statistical significance of applying a spatial score to better stratify responders vs. non-responders in this patient cohort of NSCLC.

SUMMARY & CONCLUSION

PhenoCode Signature Panels are poised to transform translational workflows in precision oncology. These panels enable rapid and systematic analysis of the TME by asking key questions related to the presence, distribution, subtype, and status or functional state of tumor and immune cells. With our "Plus One" strategy, there is built-in flexibility to answer additional questions to interrogate the TME based on YOUR markers of interest. Because the panels provide a verified, out-of-the-box solution requiring minimal assay development and optimization by users, individual labs see faster development of predictive spatial signature biomarkers that can ultimately allow for better stratification of patients and more successful treatment outcomes.

ACKNOWLEDGMENTS

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