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Profiling spatial signatures in the microenvironment of non-small cell lung carcinomas in immunotherapy responsive and resistant disease cohorts

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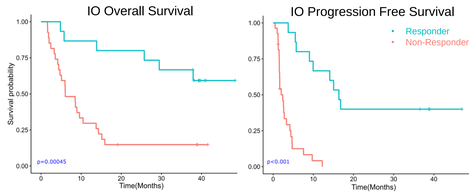
1. Introduction

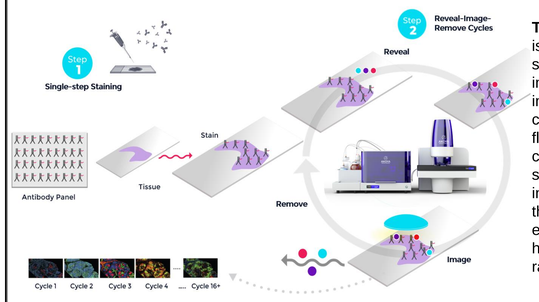
Immunotherapies have revolutionized cancer treatment. The immune contexture of the tumor microenvironment (TME) is an important factor in dictating how well a tumor responds to immune checkpoint inhibitors, but biomarker information alone is insufficient to design appropriate treatments. Instead, spatial classification of the immune contexture within the TME is foundational to addressing how immunological composition and status dictate patient responses. We used a combination of high-fidelity and high throughput spatial biomarker imaging to thoroughly characterize the TME of non-small cell lung carcinoma (NSCLC) tissue samples against outcomes for the immunotherapy arm of the Nivolumab trial. Tissues were sourced from cohorts of complete responders, partial responders, stable disease (not shown), and progressive disease patients. We first analyzed tissues using an ultra-high plex antibody panel of 46 antibodies with the PhenoCycler™-Fusion system, which provides whole-slide, single-cell resolution imaging data from FFPE samples. We then analyzed serial sections via whole-slide multiplex-immunohistochemistry (mIHC) using the PhenoImager™ HT spatial imaging platform. Here, we used six-plex antibody panels – derived from the original 46-plex panel - aimed at T-cell profiling, immune exhaustion, and immuno-modulatory targets. Both analyses revealed similar spatial signatures across cohorts, which were all derived from the immunotherapy arm of the study.

Patient cohort	Immunotherapy (n=41)
CR	2
PR	12
PD	13
SD	14
Sub-cohort in this study	**Immunotherapy (n=20)**
CR	2
PR	6
PD	5
SD	7



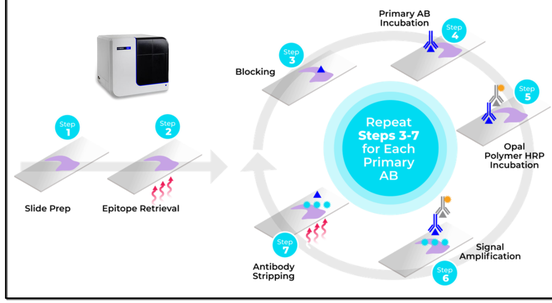
Kaplan-Meier curves of patient cohorts where CR, complete response; PR, partial response; PD, progressive disease; SD, stable disease. Non-responders are grouped for PD & SD; responders grouped for CR& PR.

2.1 PhenoCycler™-Fusion: Ultrahigh-plex Spatial Phenotyping



The PhenoCycler™-Fusion is an automated benchtop system for ultrahigh-plex immunofluorescence imaging. The CODEX®-compatible PhenoCycler fluidics device handles the cyclical oligo-based workflow shown on the left and integrates seamlessly with the PhenoImager Fusion for end-to-end spatial imaging at high resolution and with rapid turn-around time.

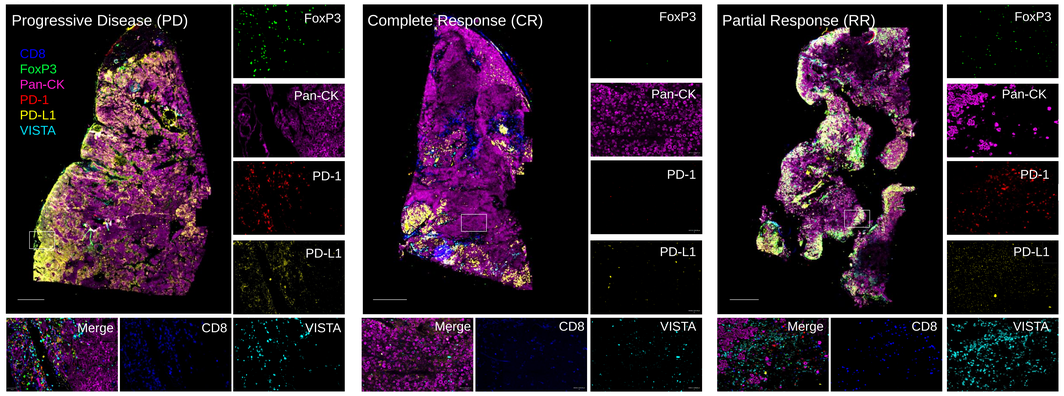
2.2 PhenoImager™ HT: High throughput Spatial Phenotyping



The **PhenoImager™ HT** instrument enables rapid whole-slide multispectral imaging when combined with Opal-Tyramide Signal Amplification (TSA) detection chemistry (illustrated in diagram). The system provides sensitivity and throughput required for spatial phenotyping at scale, i.e., as in patient cohorts in this sample.

3. Ultrahigh-plex Spatial Phenotyping of Non-small Cell Lung Cancer Tissue Cohorts

A) 46-plex Whole-slide Imaging



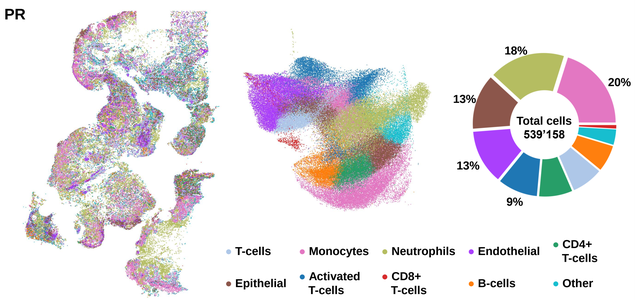
46-plex Deep Immune Profiling Panel

Immune Cell Core		Structural Module	Activity Module	Advanced Module
CD4	CD14	E-cadherin	**PD-1***	CD21
CD68*	CD56	CD31	**PD-L1***	Pax5
CD20	CD19	Podoplanin	ICOS	**FOXP3***
CD11c	*Advanced Myeloid Module*	SMA	TIM3	Gran B
CD8*		Vimentin	LAG3	CD38
HLA-DR	CD163	Collagen IV	IDO1	CD39
Ki67	CD11b	CD34	CD40	TIGIT
CD45RO	MPO	B-catenin	HLA-E	
PanCK*	iNOS		IFNG	
CD3e			VISTA	
CD44			CD15	
CD45				
HLA-A				

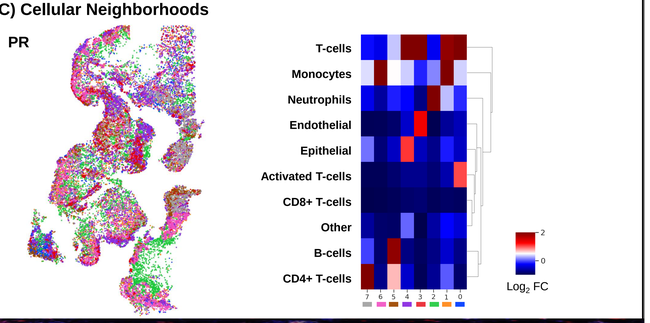
*Same markers are in MOTiF™ Panel below

A) PhenoCycler™-Fusion whole-slide 46-plex imaging of non-small cell lung carcinoma tissue from different immunotherapy cohorts (biomarkers as indicated, scale bar, 2mm). The antibody panel on the left was designed for deep-phenotyping of immune cells. **B) Single-cell Spatial Phenotyping** of 539,158 cells in an NSCLC PR tissue. Cells are phenotyped via unsupervised clustering of biomarkers. The abundance of each cell type is quantified in the accompanying pie chart. **C) Cellular neighborhoods in NSCLC tissue**. Seven different neighborhoods (colored areas) contain different proportions of immune, cancer and epithelial cells. The heatmap shows the log fold change from each cell neighborhood compared to the average of the whole tissue, which indicates the respective frequencies of different cell types within each cellular neighborhood.

B) Single Cell Spatial Phenotyping



C) Cellular Neighborhoods



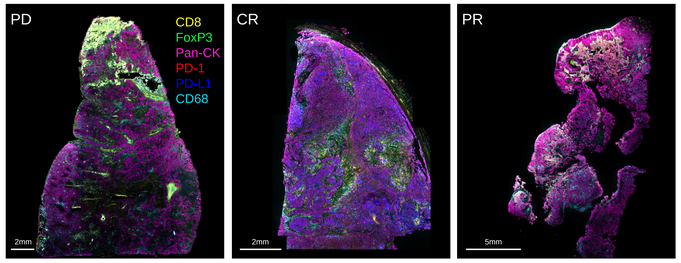
4. Multispectral Spatial Analysis Predicts Clinical Response to Immunotherapy

A) High-throughput whole-slide immunofluorescence imaging

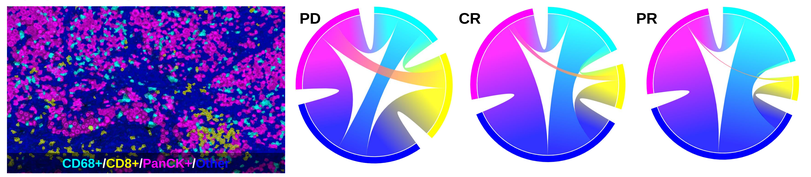
MOTiF™ Lung Cancer Kit

PD-L1	Opal 520
CD8	Opal 540
FoxP3	Opal 570
PD-1	Opal 620
Pan-CK	Opal 690
CD68	Opal 780

Serial sections from the same tissues as in Box 3 above were imaged with MOTiF™ on the PhenoImager™ HT platform.

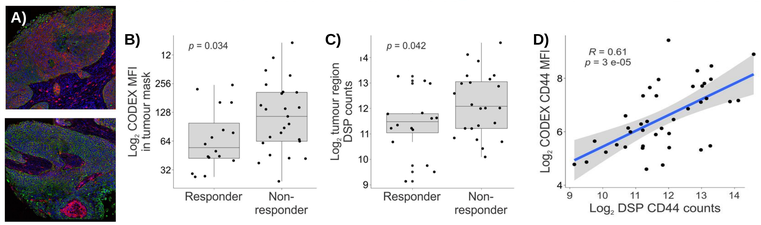


B) Spatial analysis of NSCLC tissue cohorts



A) Whole-slide scans of NSCLC tissues with MOTiF. **B) Partial immune cell neighborhood analysis**; segmentation masks show distribution of certain cell types by lineage (CD8+ T-cell, CD68+ macrophages, PanCK+ tumor/epithelia, other). Circos plots show the dynamic of nearest neighbor interactions between these cell types across cohorts. Notably, T-cells and macrophages show less direct interactions in treatment groups.

5. Correlation of Spatial Phenotyping Data with DSP Readouts



Cross-validation of tumor CD44 expression in CODEX and DSP data.
A) CD44 staining (red) in tumor cells (green) in non-responder (top) vs. responder (bottom). **B)** Geometric mean fluorescence intensity (MFI) of CD44 by CODEX in responders and non-responders. **C)** Log_2 CD44 DSP counts in responders and non-responders. **D)** Correlation between datasets.

6. Conclusions and Outlook

- **This study reports interim analysis for a multi-omic dataset from a single-agent Nivolumab arm from a clinical trial and the data are reliable across different methods, tissue cohorts and collection sites**.
- Imaging data were quantified using different approaches and resulting data were consistent with prior findings. Our data demonstrated that high-plex analysis of the TME is likely to provide new insights into therapy resistance, and sensitivity.
- Identifying the benefit of treating patients with immune checkpoint inhibitor therapy prior to treatment will ensure better outcomes. Considering that tumor tissues are highly variable and change dynamically, there is an urgent need for the development of additional and more predictive biomarkers of response to IO therapy. Spatial phenotyping represents a solution for this unmet clinical need.

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