5504: Integrating ultrahigh-plex spatial phenotyping: From discovery to clinical applications

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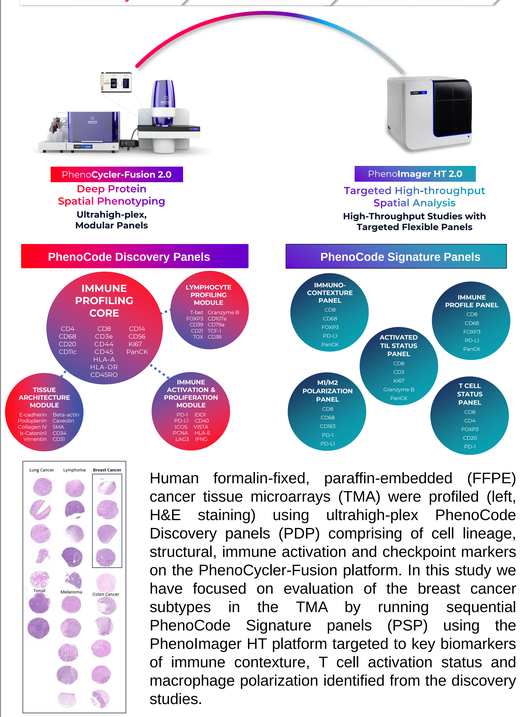
1. Introduction

The transition from ultrahigh-plex discovery experiments in multiplexed imaging to targeted, high-throughput translation and thereby clinical research studies presents a significant challenge. Successful translation hinges on the harmonization of staining, imaging, and data analysis technologies across platforms. This work aims to present a streamlined workflow for the transition, leveraging the capabilities of the PhenoCycler®-Fusion 2.0 and PhenoImager® HT platforms.

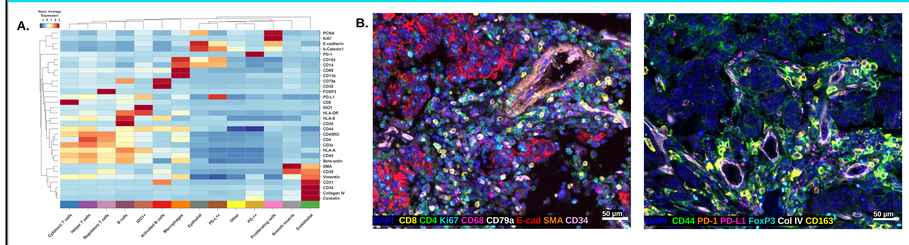
We demonstrate a two-step approach utilizing PhenoCode™ panels: 1. ***Deep Protein Spatial Phenotyping:*** For this step, tumor microarrays (TMAs) comprised of human formalin-fixed, paraffin-embedded (FFPE) cancer tissues were phenotyped using the ultra-high-plex PhenoCode Discovery panels on the PhenoCycler-Fusion 2.0 platform. This step allows for extensive characterization of the tissue microenvironment at the protein level. 2. ***Targeted High-Throughput Analysis:*** Following discovery, PhenoCode Signature panels were employed on the PhenoImager HT 2.0 platform. This platform enables high-throughput analysis of protein targets identified during the discovery phase, facilitating the translation of these findings into targeted translational and clinical research studies.

2. Discovery to Clinical Research Workflow



Human formalin-fixed, paraffin-embedded (FFPE) cancer tissue microarrays (TMA) were profiled (left, H&E staining) using ultrahigh-plex PhenoCode Discovery panels (PDP) comprising of cell lineage, structural, immune activation and checkpoint markers on the PhenoCycler-Fusion platform. In this study we have focused on evaluation of the breast cancer subtypes in the TMA by running sequential PhenoCode Signature panels (PSP) using the PhenoImager HT platform targeted to key biomarkers of immune contexture, T cell activation status and macrophage polarization identified from the discovery studies.

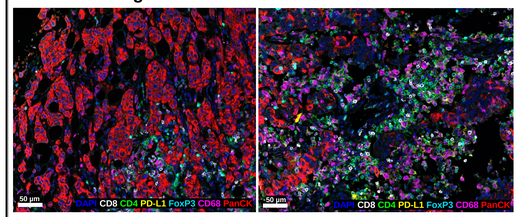
3. Ultrahigh-Plex Spatial Phenotyping of Breast Cancer Tissues

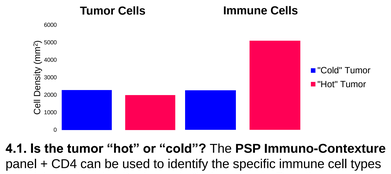


Single-Cell Spatial Phenotyping of Proteins in Breast Cancer Tissues using 32-plex Discovery Panel reveals distinct cell populations. Three types of breast cancer TMA cores: triple negative breast cancer, invasive breast carcinoma, and invasive ductal carcinoma were imaged and analyzed. Heatmap (A) corresponding to average expression of cell-type defining markers revealed 13 distinct cellular populations identified by unsupervised clustering. (B) Representative images from invasive ductal carcinoma. The difference in the immune cell composition and status in the invasive ductal carcinomas were further analyzed using 6-plex **PhenoCode Signature Panels** which are ideally suited for automated high-throughput studies.

4. Quantitative Spatial Profiling of Breast Cancer Subtypes using 6-plex Multiplex Imaging and Phenotyping Analysis

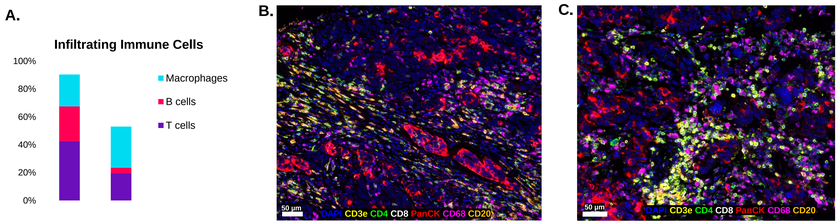
4.1. Assessing immune cells infiltration in the tumor





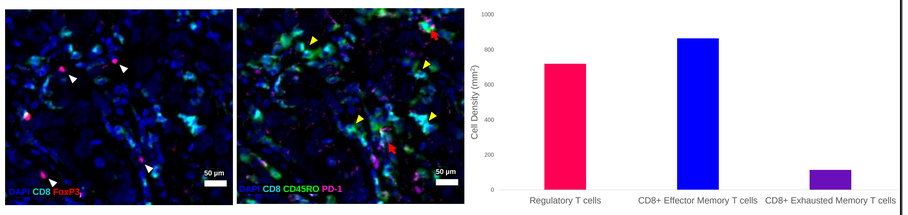
4.1. Is the tumor "hot" or "cold"? The **PSP Immuno-Contexture** panel + CD4 can be used to identify the specific immune cell types to quickly determine if a tumor is "hot" or "cold". Despite comparable numbers of tumor cells, the immune cell populations are enriched within the invasive ductal carcinoma sample relative to the triple negative breast cancer sample, indicating a "hot" tumor.

4.2. Determining major immune cell types in the tumor



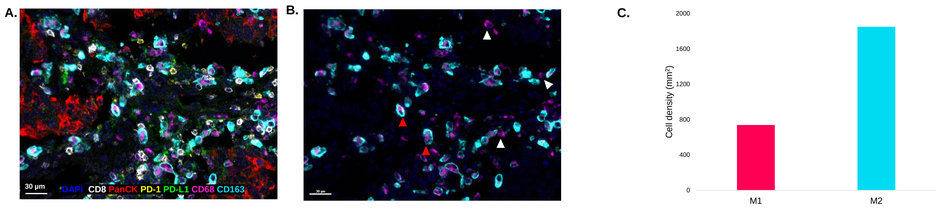
4.2. Assessment of major immune cell types in the tumor. The **PSP Immune Profile** panel + CD4 was used to quantify the major immune cell types in the different invasive ductal carcinoma samples. Assessment with the Immune Profile panel + CD4 showed differences in macrophage, B and T cell densities between the invasive ductal carcinoma samples (A). Representative images of the Immune Profile panel from the different samples are shown (B & C).

4.3. Analysis of T cell status within the tumor



4.3. Determination of T cell status. The **PSP T Cell Status** panel + CD45RO was used to phenotype exhaustive T cells**.** Regulatory T cells (CD8-/FoxP3+, white arrowhead – left image), CD8 Effector Memory T cells (CD8+/CD45RO+/PD-1-, yellow arrowhead – center image), and CD8 Exhausted Memory T cells (CD8+/CD45RO+/PD-1+, red arrow – center image) indicate the status of the T cells in an invasive ductal carcinoma sample. Cell density for the sample is shown on the right.

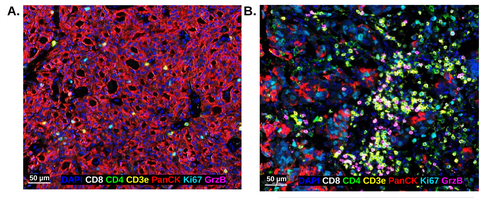
4.4. Assessing macrophage polarization in the tumor

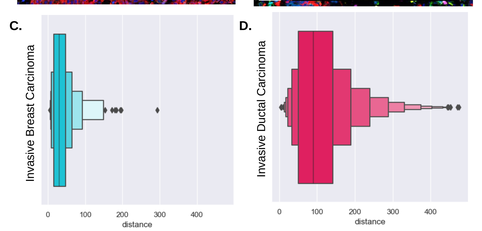


4.4. Single-cell spatial phenotyping of invasive ductal carcinoma reveals distinct macrophage polarization profiles. The **PSP M1/M2 Polarization** panel + PanCK was used following the Immune Profile panel to assess macrophage polarization in an invasive ductal carcinoma core. Representative images are shown. (A) 6-plex and (B) M1 (CD68+/CD163-, white arrowhead) & M2 (CD68+/CD163+, red arrowhead). Cell densities for the M1 and M2 macrophages are shown on the right (C).

5. Assessment of Spatial Relationships in Breast Cancer Subtypes

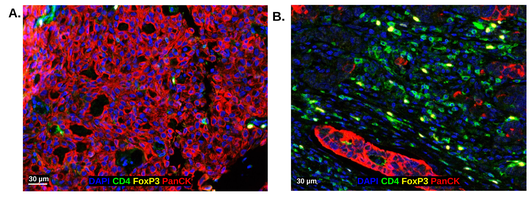
5.1. Nearest Neighborhood Analysis

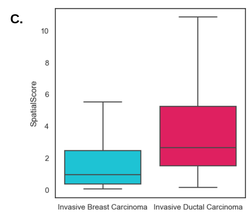




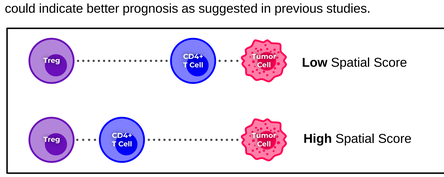
5.1. Nearest Neighbor (or spatial proximity) analysis could indicate prognosis in breast cancer. The **PSP Activated TIL** panel + CD4 was used to determine the distance between activated cytotoxic T cells (CD8+/Granzyme B+) and proliferating tumor cells (PanCK+/Ki67+). Representative images for invasive breast and ductal carcinoma are shown (A & B). Shorter distance between cytotoxic T cells and tumor cells seen in invasive breast carcinoma (C) versus invasive ductal carcinoma (D) could indicate better prognosis as suggested in previous studies.

5.2. Spatial Score Analysis





5.2. Spatial score indicates T cell suppression in invasive ductal carcinoma. The **PSP Immuno-Contexture** panel + CD4 was used to assess the distances of tumor cells and Tregs to CD4+ T Cells. Representative images of invasive breast (A) and ductal (B) carcinoma are shown. The per cell (C) spatial score (see below for spatial score details) were assessed showing a higher score in invasive ductal carcinoma samples.



The Spatial Score calculated here is the ratio of the physical distance between CD4+ T-cells and the nearest tumor cell, relative to its nearest Treg (CD4+ T Cell to Tumor Cells / CD4+ T Cell to Tregs). Tregs can exert immunosuppressive effects on CD4+ T-cells, which then results in a higher Spatial Score.

Combining these spatial analyses can provide a more in depth understanding of the TME. The invasive breast carcinoma displays a shorter distance by spatial proximity measurement than the invasive ductal carcinoma (5.1), which correlates with the spatial score results (5.2)

6. Unlocking the Power of PhenoCode Panels for Ultrahigh-plex Discovery to Targeted High-throughput Studies

PhenoCode panels, powered by Akoya's novel barcode chemistry, provide an off-the-shelf solution for immunofluorescence staining that requires minimal optimization. Our study showcases how ultrahigh-plex profiling using PhenoCode Discovery panels can be followed by a more targeted analysis using PhenoCode Signature panels. Here, we have shown how the PhenoCode Signature panels can be leveraged for rapid immune profiling and immune status assessment of the TME and to perform spatial analysis to gain key biological insights that can be used for the development of spatial signature that can more reliably predict immunotherapy response. akoyabio.com/phenocode/

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