Ultrahigh-plex Spatial Phenotyping of Cutaneous Squamous Cell Carcinoma from Immune-competent and Immunocompromised Patients

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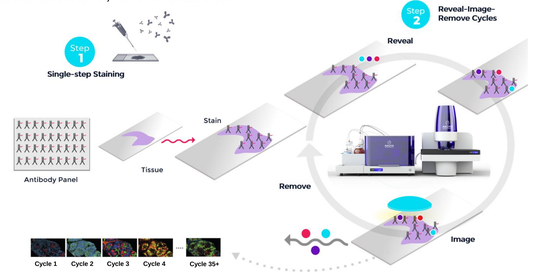
1. Ultrahigh-plex Spatial Phenotyping

Ultrahigh-plex Single-cell Spatial Phenotyping has provided valuable insights into the tumor microenvironment (TME) and will play a critical role in biomarker discovery and translational immuno-oncology research. Here, we present ultrahigh-plex spatial phenotyping of cutaneous squamous cell carcinoma with 41 markers encompassing *cell lineage*, *activation states*, *immune checkpoints*, *tissue structure, and metabolism*.

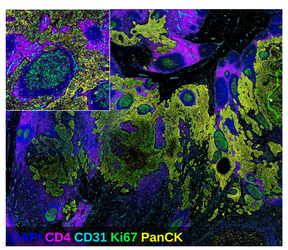


Whole-slide Spatial Phenotyping is automated on the **PhenoCycler®-Fusion**, an end-to-end integrated spatial biology platform with single-cell resolution and rapid turnaround time.

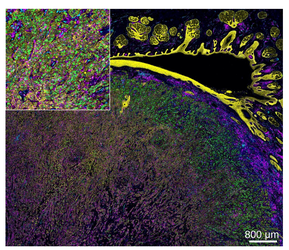
Cell Profiling Core	CD4, CD68, CD20, CD11c, CD8, HLA-DR, Ki67, CD45RO, PanCK, CD3ε, CD44, CD45, HLA-A, CD14, CD57, CD19
Adv. Immune Module	CD21, FOXP3, Granzyme B, CD163, CD79α, PD-1, PD-L1, IDO1
Structural Module	E-cadherin, CD31, Podoplanin, SMA, Vimentin, Collagen IV, CD34
Metabolic Module	**ASCT2, CPT1A, HK1, LDHA, IDH2, GLUT1, pNRF2, ATP5A, Citrate Synthase, HIF1α**

2. Cutaneous Squamous Cell Carcinoma (cSCC)

Cutaneous squamous cell carcinoma is the second most common non-melanoma skin cancer. Though prognoses are favorable in most cases, locally advanced and metastatic forms present an emerging health burden. Immunotherapy is a promising solution; however, resistance to immune checkpoint inhibitors (ICI) warrants more research into tumor biology to identify predictors of response and resistance. To that end, *we developed a novel antibody module of 10 metabolic and stress markers* to further elucidate the tumor microenvironment in metastatic and recurrent cases.



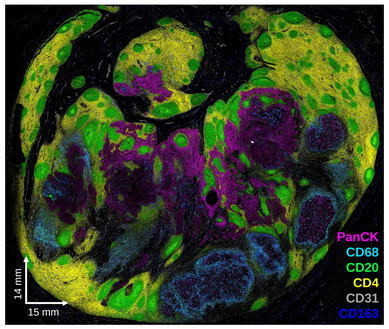
Immuno-competent cSCC with Regional Lymph Node Metastasis

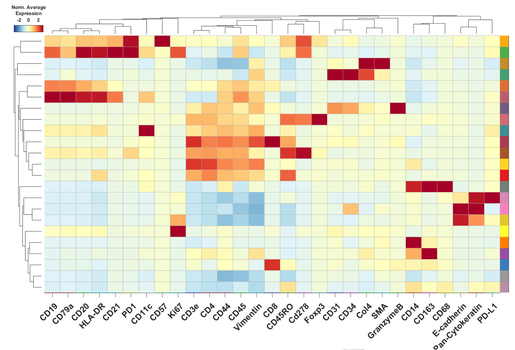


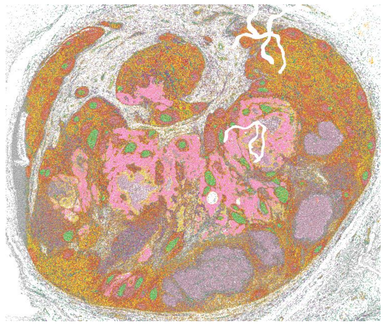
Recurrent/Metastatic Disease treated with Immunotherapy

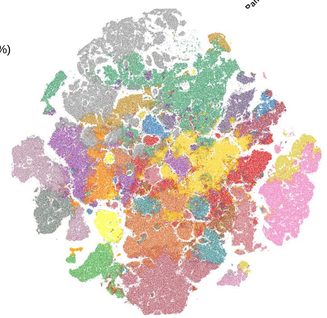
3. Whole-slide Spatial Phenotyping of cSCC with Regional Lymph Node Metastasis Reveals an Immune-competent Microenvironment

3.1 Spatial Phenotyping Reveals 23 Distinct Cell Phenotypes in cSCC with Lymph Node Metastasis





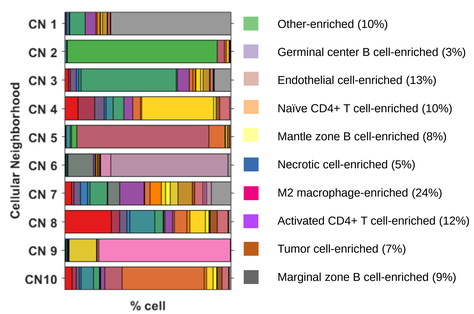


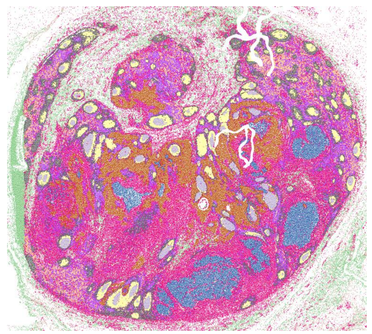


- Activated CD4+ T cells (6%)
- Activated CD8+ T cells (2%)
- CD4+ cytotoxic T lymphocytes (3%)
- CD8+ T cells (2%)
- Dendritic cells (4%)
- Endothelial cells (12%)
- Germinal center B cells (3%)
- M1 macrophages (3%)
- M2 macrophages (6%)
- Mantle zone B cells (8%)
- Marginal zone B cells (8%)
- Monocytes (2%)
- Natural killer (NK) cells (<1%)
- Naïve CD4+ T cells (7%)
- Proliferating cells (2%)
- Proliferating tumor cells (2%)
- Smooth muscle cells (3%)
- T follicular helper (Tfh) cells (2%)
- T regulatory cells (3%)
- Tumor cells (6%)
- PD-L1+ tumor cells (<1%)
- Necrotic cells (5%)
- Other (12%)

3.1 Whole Slide Imaging of a cSCC with metastasis to the lymph node (top left; biomarkers as indicated; FFPE tissue). **Spatial Phenotyping** (top right) of 1.4 million cells reveals 23 cell types based on expression of 29 biomarkers (see panel to the left). The heatmap includes a curated clustering dendrogram with cell types indicated with colors as in the legend below the heatmap. A **Spatial Phenotyping Map** (bottom left) of the entire tissue section demonstrates the overall topography and organization of the different cell types relative to each other. A **tSNE Plot** (bottom right) illustrates the abundance of distinct phenotypic clusters sorted by color and geography.

3.2 Cellular Neighborhood (CN) Analysis Reveals 10 Unique Neighborhoods with High Immune Infiltration

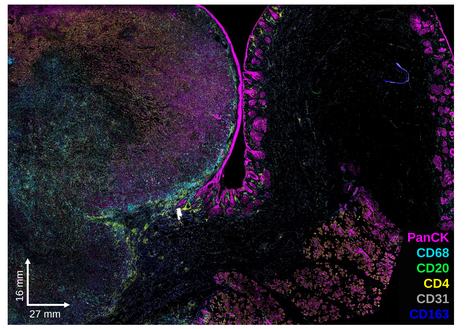


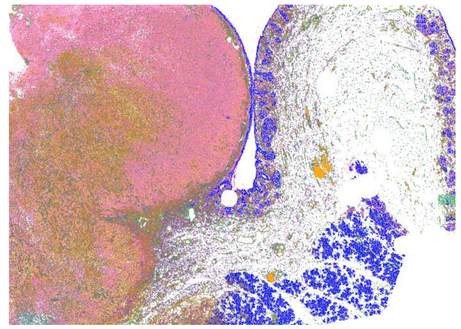


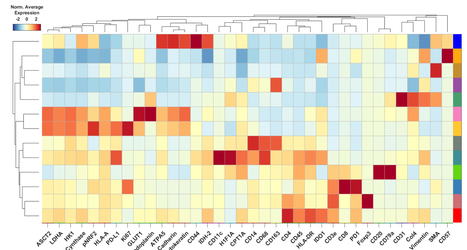
3.2 Cellular Neighborhood Analysis reveals 10 unique spatial neighborhoods enriched in specific cell types and varying in abundance as shown in the legend. The accompanying bar chart (left) shows the percentage of each cell type (colored as per the legend in 3.1) within the CN. A **Spatial Neighborhood Map** (right) further illustrates the geographical distribution of the CNs across the entire tissue section. **The extreme degree of immune infiltration in this tumor suggests an immune-responsive microenvironment that may produce a favorable response to immunotherapy.**

4. Whole-slide Spatial Phenotyping of Recurrent and Metastatic cSCC Treated with Immunotherapy Reveals a Metabolically Active and Resistant Microenvironment

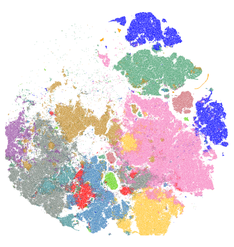
4.1 Spatial Phenotyping Reveals 13 Distinct Phenotypes in Recurrent and Metastatic cSCC





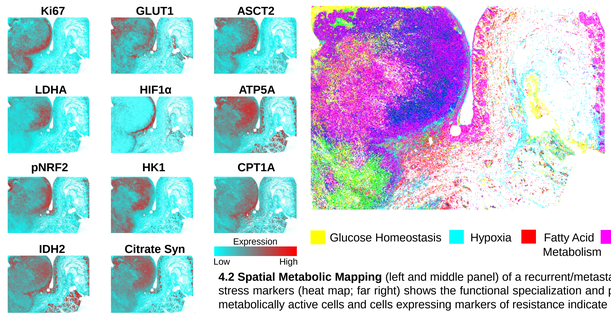


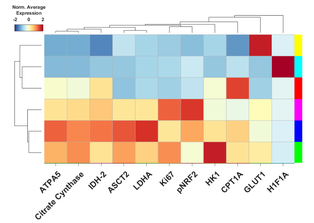
- Epithelial cells (11%)
- Natural killer (NK) cells (<1%)
- Smooth muscles (9%)
- M2 macrophages (3%)
- Endothelial cells (12%)
- Tumor cells (24%)
- Proliferating tumor cells (9%)
- M1 macrophages (14%)
- Dendritic cells (3%)
- B cells (1%)
- CD8+ T cells (8%)
- T regulatory cells (2%)
- CD4+ T cells (3%)



4.1 Whole Slide Imaging of the Tumor-Immune Microenvironment at Single Cell Resolution in a FFPE recurrent and metastatic cSCC (top left; biomarkers as indicated; FFPE tissue). **Spatial Phenotyping** of all 1.6 million cells in the tissue into 13 distinct cell types based on expression of 36 cellular phenotypic markers as shown in the heat map (bottom left). A **Spatial Map** and **tSNE Plot** show the geographical and numerical distribution of the distinct cell types.

4.2 Deep Spatial Phenotyping based on Metabolic Markers Reveals High Metabolic Activity and Resistance to Oxidative Stress





Glucose Homeostasis | Hypoxia | Fatty Acid Metabolism | Oxidative Stress and Resistance | Metabolic Activation | Glycolysis

4.2 Spatial Metabolic Mapping (left and middle panel) of a recurrent/metastatic cSCC tumor based on expression of key metabolic and stress markers (heat map; far right) shows the functional specialization and predominant pathways across the entire tissue. Clusters of metabolically active cells and cells expressing markers of resistance indicate a highly proliferative environment.

5. The Value of Deep, Unbiased Spatial Analysis for Tumor-Immune Phenotyping

This study demonstrates the power of rapid, deep single-cell spatial phenotyping enabled by the PhenoCycler-Fusion system for a comprehensive analysis of the TiME and tumor metabolome. Understanding differences in the tumor microenvironment at multiple levels – immune, metabolic and stress – will be crucial for improving patient stratification and clinical care.

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