6872: A Spatio-Temporal Approach to Mapping the Dynamics of Cutaneous Squamous Cell Carcinoma Progression and Immunotherapy Response: A Journey through TiME

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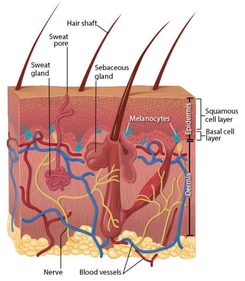
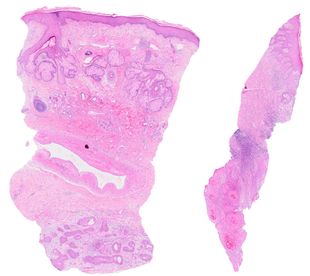
1. Cutaneous Squamous Cell Carcinoma

Cutaneous Squamous Cell Carcinoma (cSCC), arising from proliferation of malignant epidermal keratinocytes, is the *second most common non-melanoma skin cancer*[1]. Tumor development is a gradual process characterized by a high *mutational burden* and an *immunosuppressive* tumor immune microenvironment (TiME). Though **immunotherapy** is a promising solution, and prognoses are favorable in most cases with a 5-year survival ≥90%, cSCC accounts for 75% of all skin-cancer related deaths excluding melanoma[1].

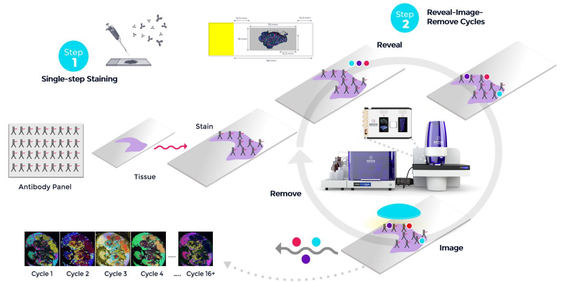




[1]Fania et al. Biomedicines. 2021 Feb 9;9(2):171; Illustration: Centers for Disease Control and Prevention (CDC)

2. PhenoCycler-Fusion 2.0 Technology

The PhenoCycler®-Fusion 2.0 (PCF) technology is the fastest whole-slide spatial biology platform that enables simultaneous detection of 100+ biomarkers by combining automated fluidics and iterative imaging of DNA-tagged antibodies. Leveraging the high plex, high throughput single-cell resolution of PCF, we sought to identify the **spatio-temporal changes** in the cSCC microenvironment. In this interim analysis, we profiled two patients before immunotherapy with Cemiplimib and 6-12 months following treatment.



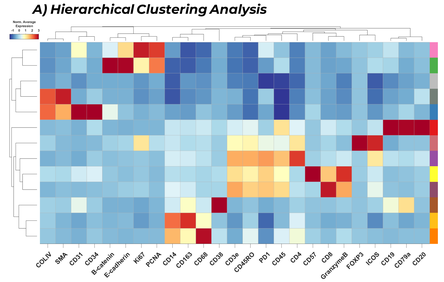
PhenoCode™ Discovery Panels

Cell Profiling Core	CD4, CD68, CD20, CD11c, CD8, HLA-DR, Ki67, CD45RO, CD3ε, PanCK, CD44, CD45, HLA-A, CD14, CD56 + CD163, CD11b, CD57
Lymphocyte Profiling Module	CD21, FOXP3, Granzyme B, CD79α, TCF-1, CD38, TOX, T-bet, CD107α, CD39
Immune Activation and Proliferation Module	PD-1, PD-L1, IDO1, LAG3, VISTA, IFNG, HLA-E, CD40, ICOS, PCNA
Tissue Architecture Module	E-cadherin, CD31, Podoplanin, SMA, Vimentin, Collagen IV, CD34, β-catenin1, β-actin, Caveolin
Custom Metabolic Module	ASCT2, CPT1A, HK1, LDHA, IDH2, GLUT1, pNRF2, ATP5A, Citrate Synthase, HIF1α, G6PD, SDHA

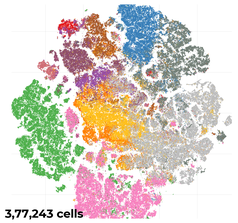
3. Spatio-Temporal Analysis Reveals Differential Tumor-Immune Phenotypes

3.1 Whole-Slide Single-Cell Spatial Phenotyping Identifies 12 Distinct Cellular Phenotypes in cSCC Tissues

A) Hierarchical Clustering Analysis



B) t-SNE Plot



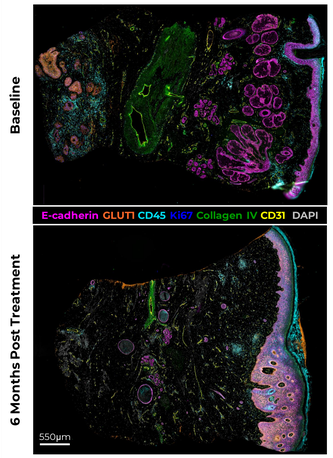
- B cells
- CD8+ T cells
- Vasculature
- Fibroblasts
- CD4+ T cells
- M1 macs
- M2 macs
- NKT cells
- Plasma cells
- Tregs
- Tumor cells
- Epithelial cells
- Other

A) Heatmaps with Cellular Phenotypes (colored as shown in the legend on the right) annotated by hierarchical clustering analysis and expression of cell lineage and structural markers from the **PhenoCode Discovery Panels**.

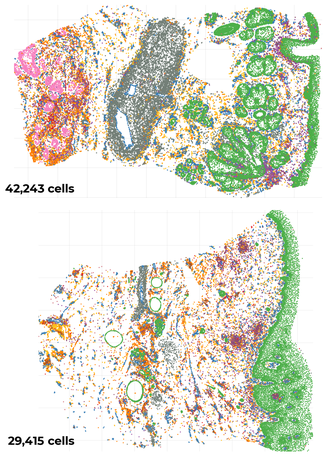
B) t-SNE Plot showing the multiplex data and distinct cellular phenotypes in reduced dimensionality.

3.2 Spatio-Temporal Analysis Reveals a "Responder" Phenotype with Tumor Regression and M1 Polarization

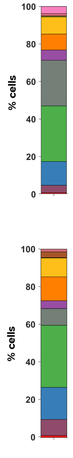
A) Whole-slide Multiplex PCF Image



B) Single-cell Spatial Map



C) Quantification



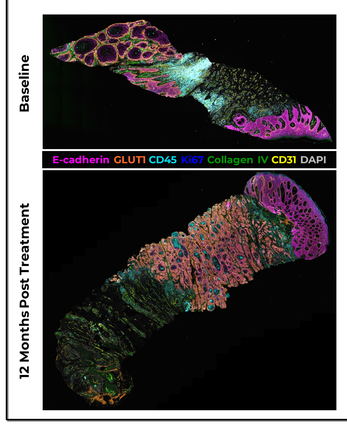
A) Whole-Slide Multiplex PCF Images of a cSCC, labeled with epithelial (E-cadherin), vascular (CD31, CollV), immune (CD45), metabolic (GLUT1) and proliferation (Ki67) markers at baseline and 6 months post treatment, reveal the heterogeneity in spatial landscapes across the course of treatment in the same patient.

B) Single-Cell Spatial Maps illustrate the differences in the distribution and spatial organization of the twelve distinct cellular phenotypes (legend in section #3.1). A marked difference is observed in overall tumor burden.

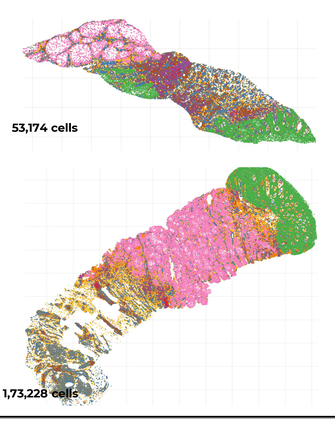
C) Bar Charts illustrate the abundance of the different phenotypes. Higher percentages of M1 macrophages (orange), CD8+ T cells (dark purple) and plasma cells (brown) are seen post treatment, correlating with regression of the tumor (pink).

3.3 Spatio-Temporal Analysis Reveals a "Non-Responder" Phenotype with Tumor Burden and M2 Polarization

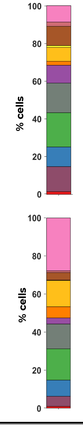
A) Whole-slide Multiplex PCF Image



B) Single-cell Spatial Map



C) Quantification



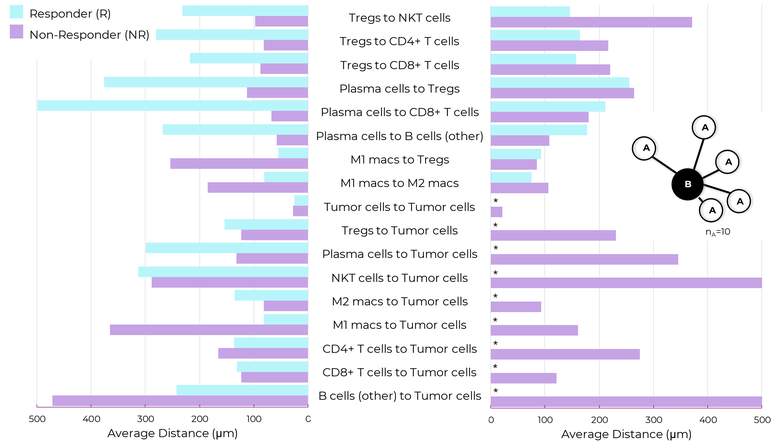
A) Whole-Slide Multiplex PCF Images of a cSCC, labeled with epithelial (E-cadherin), vascular (CD31, CollV), immune (CD45), metabolic (GLUT1) and proliferation (Ki67) markers at baseline and 12 months post treatment, reveal the heterogeneity in spatial landscapes across the course of treatment in the same patient.

B) Single-Cell Spatial Maps illustrate the differences in the spatial organization of the twelve distinct cellular phenotypes (legend in section #3.1). A marked increase is observed in overall tumor burden.

C) Bar Charts illustrate the abundance of the different phenotypes. Higher percentages of M2 macrophages (mustard yellow) along with a decrease in CD8+ T cells (dark purple) and plasma cells (brown) post treatment correlates with tumor proliferation (pink).

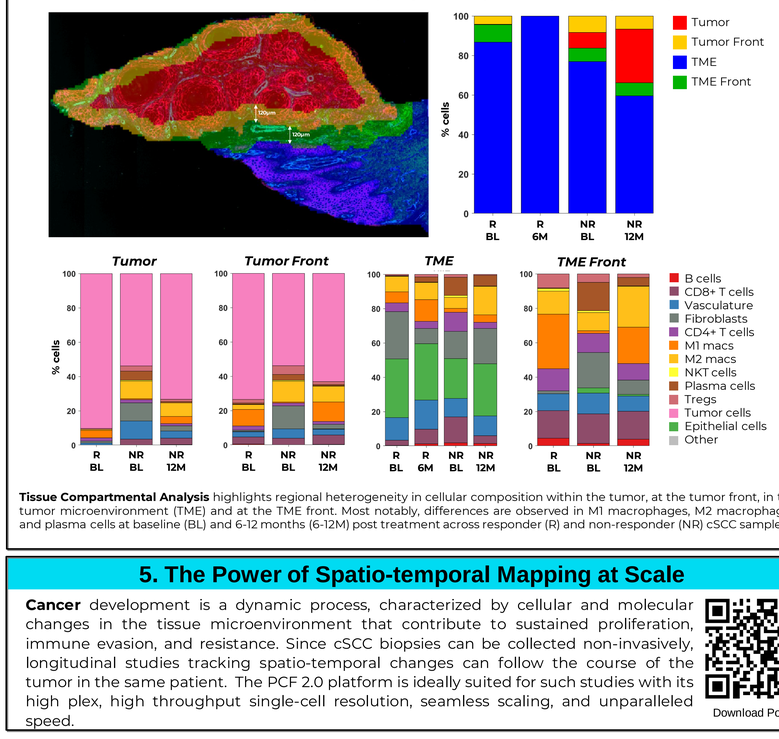
4. Spatial Analyses Reveals Regional Heterogeneity

4.1 Spatial Proximity Analyses Reveals Temporal Differences in Cellular Distribution



Spatial Proximity Analysis shows the localization of cellular phenotypes relative to each other by measuring distances of 10 neighboring "A" cells from a focal "B" cell. Differences in cellular distribution emerge between the responder and non-responder at baseline as well as temporally within the same patient (* denotes not applicable).

4.2 Tissue Compartmental Analysis Highlights Regional Heterogeneity



Tissue Compartmental Analysis highlights regional heterogeneity in cellular composition within the tumor, at the tumor front, in the tumor microenvironment (TME) and at the TME front. Most notably, differences are observed in M1 macrophages, M2 macrophages and plasma cells at baseline (BL) and 6-12 months (6-12M) post treatment across responder (R) and non-responder (NR) cSCC samples.

5. The Power of Spatio-temporal Mapping at Scale

Cancer development is a dynamic process, characterized by cellular and molecular changes in the tissue microenvironment that contribute to sustained proliferation, immune evasion, and resistance. Since cSCC biopsies can be collected non-invasively, longitudinal studies tracking spatio-temporal changes can follow the course of the tumor in the same patient. The PCF 2.0 platform is ideally suited for such studies with its high plex, high throughput single-cell resolution, seamless scaling, and unparalleled speed.

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