Spatio-temporal Monitoring of the Tumor Microenvironment in Cutaneous Squamous Cell Carcinoma

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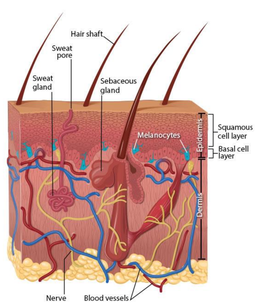
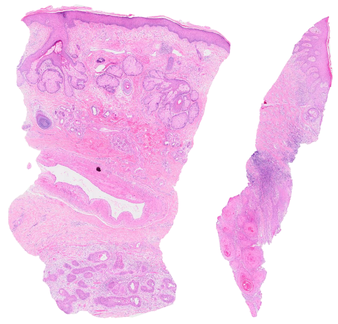
1. Cutaneous Squamous Cell Carcinoma

Cutaneous Squamous Cell Carcinoma (cSCC) is the *second most common non-melanoma skin cancer*[1]. Though prognoses are favorable in most cases, with a 5-year survival ≥90%, cSCC accounts for 75% of all deaths due to skin cancer excluding melanoma[1]. Tumor development is a gradual process characterized by a *high mutational burden* and an *immunosuppressive microenvironment*.

Immunotherapy is a promising solution; however locally advanced and metastatic forms along with resistance to immune checkpoint inhibitors (ICI) presents an emerging health burden. Identifying the molecular mechanisms of tumor progression and the underlying cellular changes in the microenvironment will be valuable to advance our understanding of cSCC biology and develop therapeutic strategies for improved management of the disease.



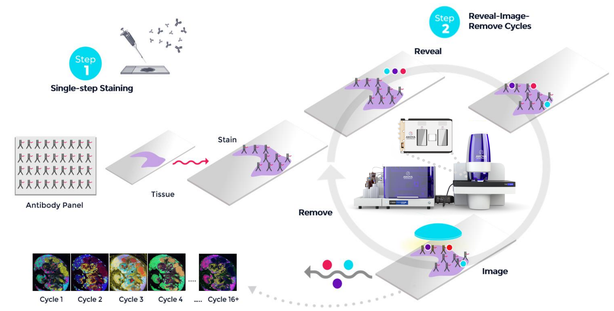


cSCC arises from malignant proliferation of epidermal keratinocytes and can invade into the dermis at various depths.

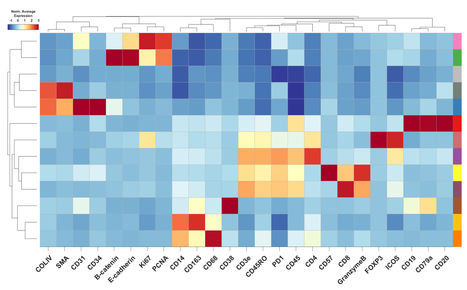
[1]*Fania et al., Biomedicines. 2021 Feb 9;9(2):171; Illustration: Centers for Disease Control and Prevention (CDC)*

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 oligo-conjugated antibodies. Leveraging the high plex, resolution and throughput of PCF, our study sought to identify the **spatio-temporal changes** in the cSCC microenvironment over the course of immunotherapy. In this interim analysis, we have profiled 2 patients pre-immunotherapy (Cemiplimib) and at follow-up after 6-12 months.



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, CD107a, 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
Metabolic Module	**ASCT2, CPT1A, HK1, LDHA, IDH2, GLUT1, pNRF2, ATP5A, Citrate Synthase, HIF1α, G6PD, SDHA**

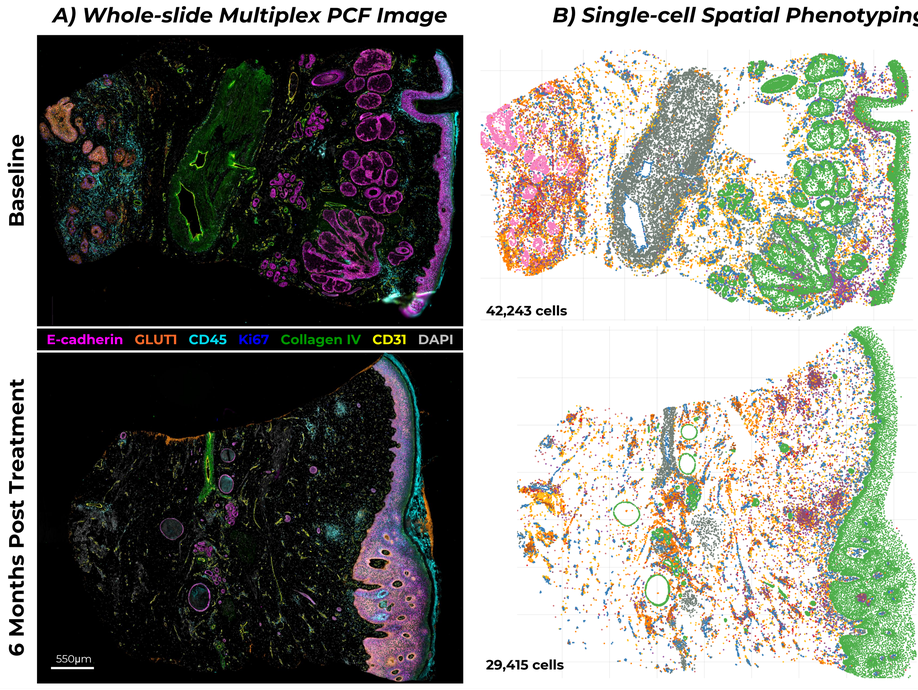


B cells, CD8+ T cells, M2 macrophages, M1 macrophages, NKT cells, Vasculature, Epithelial cells, T regulatory cells, Tumor cells, Fibroblasts, Plasma cells, CD4+ T cells, Other

Phenotypes are defined by unbiased hierarchical clustering analysis based on expression of cell lineage and structural markers from the **PhenoCode™ Discovery Panels**.

3. Spatio-Temporal Immune and Metabolic Analyses of a cSCC Reveals a "Responder" Phenotype with Tumor Regression

A) Whole-slide Multiplex PCF Image | *B) Single-cell Spatial Phenotyping* | *C) Cell-type Metabolic Expression*



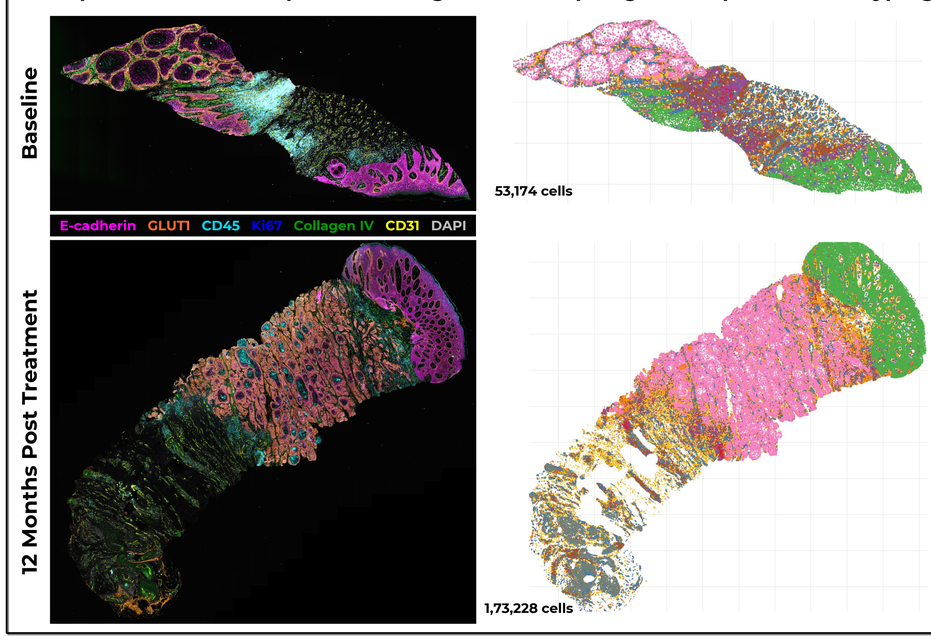
A) Whole-Slide Multiplex PCF Images of a cSCC, labeled with epithelial (E-cadherin), vascular (CD31, ColIV), immune (CD45), metabolic (GLUT1) and proliferation (Ki67) markers at baseline and 6 months post treatment, reveal the heterogeneity in spatial landscapes across the course of treatment in the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

B) Single-Cell Spatial Maps (with accompanying bar charts) illustrate the differences in the abundance and organization of 12 distinct cellular phenotypes identified by unbiased Leiden clustering (legend in section #2). Higher percentages of M1 macs (orange), CD8+ T cells (dark purple) and plasma cells (brown) are seen post treatment, correlating with tumor regression (pink).

C) Heatmaps with Expression of Metabolic Markers from TCA cycle, lipid metabolism, amino acid metabolism, glycolysis, and stress responses reveal the differential functional phenotypes across the major cell types.

4. Spatio-Temporal Immune and Metabolic Analyses of a cSCC Reveals a "Non-Responder" Phenotype with High Tumor Proliferation

A) Whole-slide Multiplex PCF Image | *B) Single-cell Spatial Phenotyping* | *C) Cell-type Metabolic Expression*



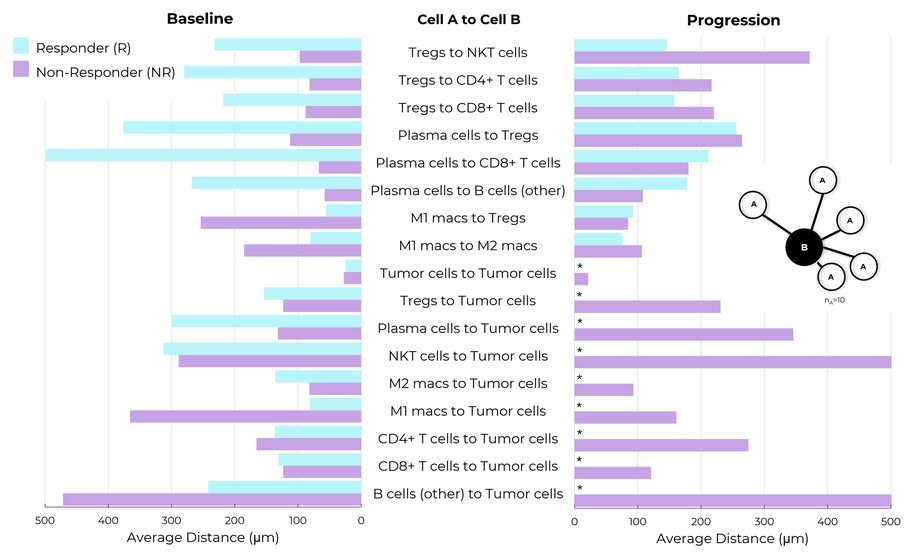
A) Whole-Slide Multiplex PCF Images of a cSCC, labeled with epithelial (E-cadherin), vascular (CD31, ColIV), immune (CD45), metabolic (GLUT1) and proliferation (Ki67) markers at baseline and 12 months post treatment, reveal the heterogeneity in spatial landscapes across the course of treatment in the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

B) Single-Cell Spatial Maps (with accompanying bar charts) illustrate the differences in the abundance and organization of 12 distinct cellular phenotypes identified by unbiased Leiden clustering (legend in section #2). Higher percentages of M2 macs (mustard yellow) along with a decrease in CD8+ T cells (dark purple) and plasma cells (brown) post treatment correlates with tumor proliferation (pink).

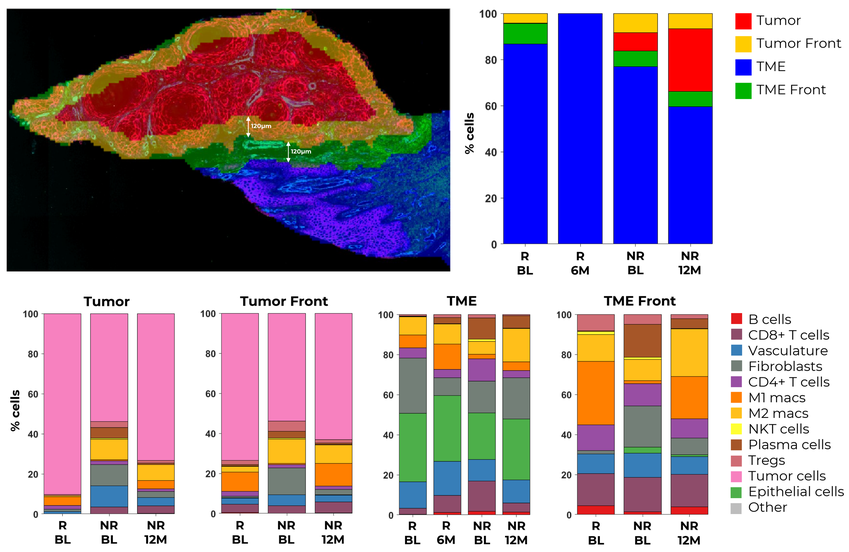
C) Heatmaps with Expression of Metabolic Markers from TCA cycle, lipid metabolism, amino acid metabolism, glycolysis, and stress responses reveal the differential functional phenotypes across the major cell types.

5. Spatial Analyses Reveals Regional Heterogeneity and Differential Organization of Cellular Phenotypes

A) Spatial Proximity Analysis



B) Tissue Compartmental Analysis



A) 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). These differences are also accompanied by regional heterogeneity in cellular composition, most notably in M1 macs, M2 macs and plasma cells across the tissue.

6. Value of Ultrahigh-Plex Spatial Phenotyping for Studying the Dynamics of cSCC Progression

Cancer development is a dynamic process, characterized by cellular and molecular changes in the tissue microenvironment that contribute to sustained proliferation, evasion of immune responses, metabolic deregulation, invasion and therapeutic resistance. Since cSCC biopsies can be collected relatively non-invasively, longitudinal studies tracking spatial and temporal changes can be carried out to follow the course of the tumor in the same patient. The PhenoCycler-Fusion 2.0 platform is ideally suited for such studies with its high throughput, high resolution, ultrahigh-plex scale and unparalleled speed.

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