Development and application of an end-to-end staining and analysis pipeline to identify immune cell infiltrates in oral cancer samples using a targeted multiplex immunohistochemistry antibody panel

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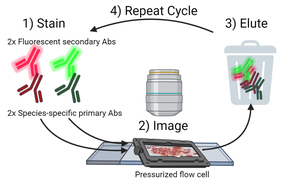
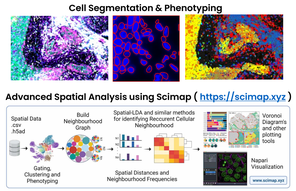
Introduction

The tumor microenvironment (TME) plays a critical role in determining how a tumor may respond to therapy, particularly for cancer immunotherapy approaches. Improvements in the number of biomarkers that can be simultaneously screened have led to the ability to probe for numerous cell types expressing multiple markers within the TME. The ability to probe the tumor microenvironment (TME) at the single cell level is important for understanding of interactions between tumors and immune cells, particularly whether immune cells have infiltrated into tumor nests, and with regards to the presence or absence of immune checkpoint markers. The ability to interrogate these questions relies on the ability to assess multiple biomarkers simultaneously for cell phenotyping in their spatial context. Here we demonstrate rapid design and optimization of a panel of antibodies for multiplexed immunohistochemical staining of a series of oral cancer tumor samples.

General Workflow: Staining



General Workflow: Analysis



Methods

Biomarkers of importance in the assessment of cancer immunotherapy status for oral cancer were identified, and a panel of antibodies was designed (Table 1). Staining was achieved using Bethyl Laboratories IHC-validated primary and secondary antibodies. Immunostaining was performed in a sequential immunofluorescence platform, the Lunaphore COMET® (Lunaphore, Lausanne, Switzerland). Simultaneous imaging of serial dilutions of antibodies in the same staining run permitted the fine tuning of the panel to provide appropriate signal coverage more rapidly than with previous multiplex panel methods. Once images were acquired, tumor nests were manually annotated, followed by application of deep-learning based StarDist segmentation to the tissue. Cellular phenotypes were identified based on marker thresholding and validated by visual inspection. Spatial analysis was performed with the help of the Scimap package (https://scimap.xyz).

	Antibody	Clone	Catalog #	Species	Significance
Immune Marker Panel	CD3	BL-298-5D12	A700-016	Rb	T-cell
	CD20	L26	A500-017A	Mo	B-cell
	FoxP3	BLR034F	A700-034	Rb	T-regulatory
	CD8	C8/144B	A500-021A	Mo	T-cyto
	PD-L1	BLR020E	A700-020	Rb	Checkpoint
	Pan-CK	AE1/AE3	A500-019A	Mo	Epithelium
	PD-1	BLR076G	A700-076	Rb	T-cell
	CD68	KP1	A500-018	Mo	Macrophages
	CD45	BL-178-12C7	A700-012	Rb	Lymphocytes
	PCNA	PC10	A500-024A	Mo	Proliferation
	CD4	BL-155-1C11	A700-015	Rb	T-helper
	Granzyme B	BLR022E	A700-022	Rb	T-activation
	CD45RO	UCHL-1	A500-020A	Mo	T-memory

Table 1. Selection of IHC-validated antibodies used in the multiplex immunofluorescence studies.

Process

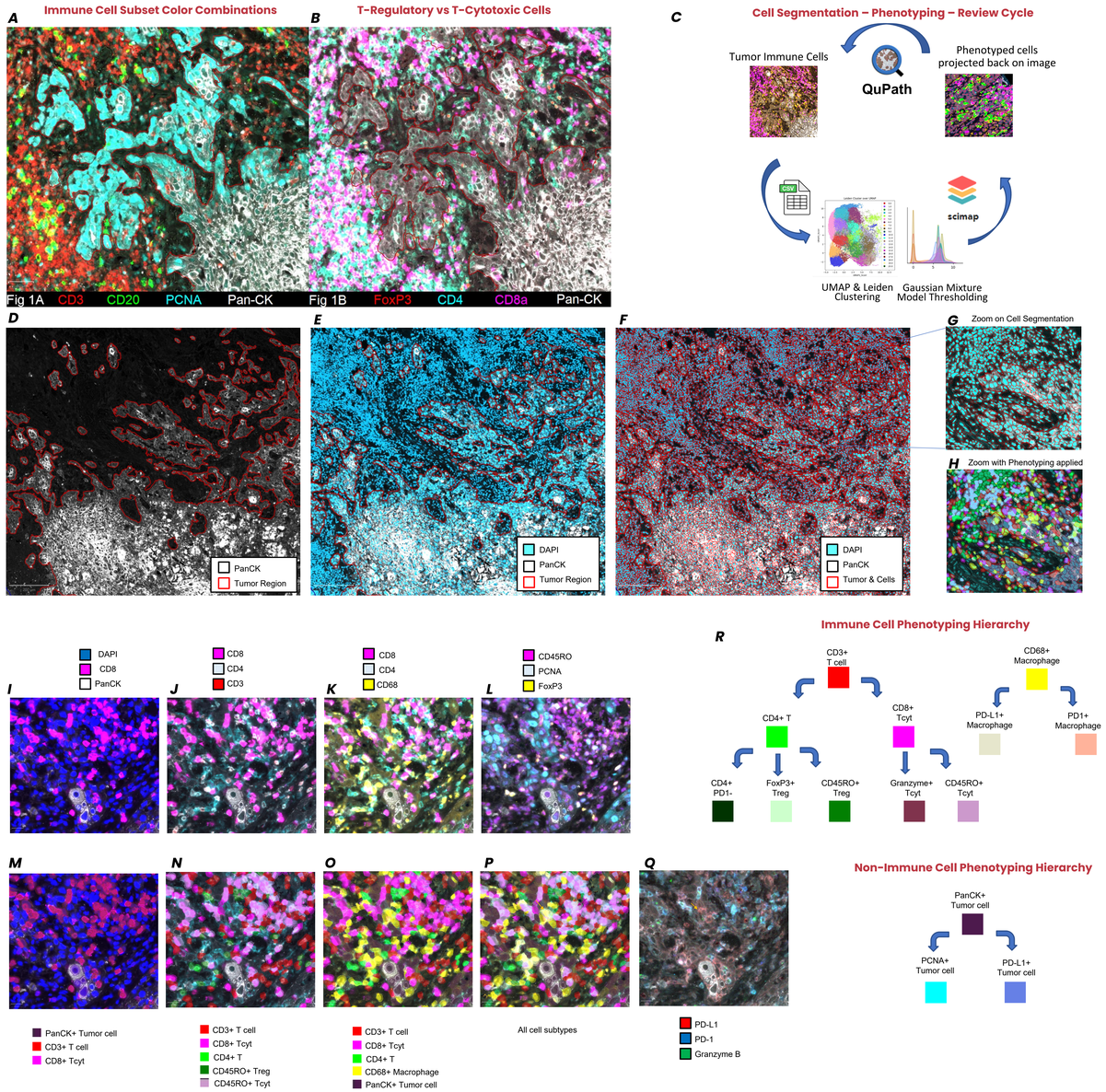


Figure 1. Establishment of a multiplex immunofluorescent assay to detect 13 biomarkers simultaneously. Cyclic mIF staining was performed on the Lunaphore COMET® system, and the whole slide images were evaluated using QuPath, with spatial analysis performed using Scimap. (A) Representative images showing combinations of markers indicating T vs B cells, and proliferating tumor cells. (B) Representative color combinations to identify T-regulatory from T-cytotoxic subsets in the same region. (C) Diagram of the process of cell segmentation, phenotyping in Scimap using Gaussian Mixture Model to identify thresholds, and then application of those cell phenotypes back onto the image, to review image intensities. (D) Tumor nests were identified using the Pan-cytokeratin as a guide, to separate melanoma tumor nests from surrounding tumor stroma. (E) The DAPI signal in the image was used to perform (F) cell segmentation using the StarDist algorithm, which was applied across the image, with a zoom in (G) to indicate segmentation detail. (H) Post-phenotyping results in the same region, using the Phenotyping Hierarchy shown in (R). The same individual zoomed in region is shown in (I-L & Q) with different channels highlighted, corresponding to the segmented and phenotyped cells shown in (M-P), from single-marker to multi-marker combinations. (R) indicates the immune cell phenotyping hierarchy applied to these images: (A,B,G,H). Scale bar: 50µm (D-F), 200µm (I-Q), 20µm

Results

Validation and optimization of thirteen unique immune markers was completed in less than two weeks. The staining and imaging of twenty-five oral cancer tissue samples was completed in five weeks and whole-slide image data was exported for analysis. Tissue and cell segmentation permitted the quantification of biomarkers in a spatially resolved manner. Quantitative analysis revealed the presence of immune cells both surrounding and infiltrating tumor nests within oral cancer tissue sections. Breaking down the immune cell types into subcategories, the phenotypes assessed included tumor cell count (PanCK+), tumor cell proliferation (CK+PCNA+), functional Th (CD3+CD4+FoxP3-PD1-), Treg; immune suppression (CD3+FoxP3+), Activated Tc (CD3+CD8a+Granzyme B+), Memory Th cells (CD3+CD4+CD45RO+), Memory Tc cells (CD3+CD8+CD45RO+). The full table for marker phenotyping is shown in Table 1, representative markers in Fig 1A/B.

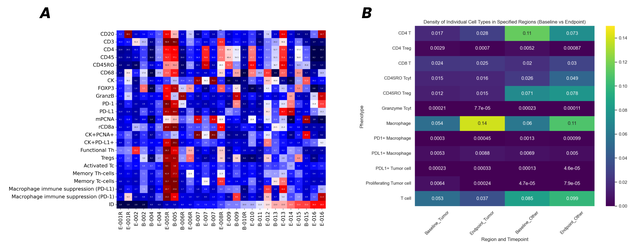


Figure 2. (A) Example phenotypic analysis (% positivity of cells) across several tissue slides from Baseline (B) and Endpoint (E) samples. The percentage of cells positive for Individual markers, as well as combinations of markers that identify specific cell types, are shown. (B) Analysis was performed by grouping images into Baseline versus Endpoint, and Intra-Tumor vs Stroma/Other, to assess tumor Infiltrating lymphocyte abundance. An increase in macrophages and decrease in proliferating tumor cells at endpoint is noted.

***SpatialScore* distance ratio = Right / Left**

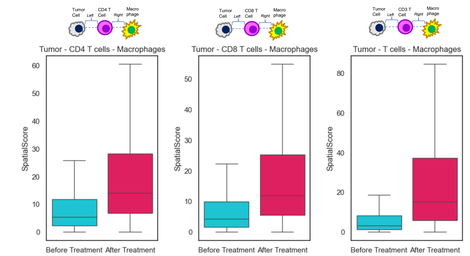


Figure 3. Spatial Immunoscore for the relative distance between cancer cells, CD8 T cells, and macrophages, showing changes induced between before-treatment and on-treatment samples.

Conclusions

Here, we present an end-to-end workflow for the rapid development of multiplexed immunostaining on clinical tissue samples. Sequential immunofluorescence staining permitted us to optimize antibody concentrations rapidly, enabling quick imaging turnaround. Quantitative image analysis was applied to identify discrete cells and define their localization relative to tumor nests, revealing areas of tumor cell proliferation and immune cell exclusion from the tumor center (Fig1A/B). The ability to provide quantitative readouts of spatial interactions allowed for a more refined understanding of tumor-immune cell interactions, permitting a more complete interrogation of the TME in a spatially resolved manner.

Acknowledgements

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