

Validation of a Multiplex Immunofluorescent Technique for Characterization of Immune Cell Populations in Head and Neck Squamous Cell Carcinoma (HNSCC)

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Objectives

Background:

- The HNSCC tumor immune microenvironment plays an important role in disease progression and response to therapy^{1,2}
- Checkpoint inhibitors are approved for recurrent HNSCC in PDL-1/PD-1+ positive subgroups
- Overall, response rates to checkpoint inhibitors remain low, and initial responders often acquire resistance^{3,4}
- The concentration of certain immune cells predict recurrence⁵
 - FoxP3+ Regulatory T cell (Tregs, FoxP3+), CD33+IL4+ Myeloid-derived Suppressor Cells (MDSCs)
- PDE5 inhibitor therapy with Tadalafil lowers MDSCs and Tregs in blood and tumor of HNSCC patients while increasing tumor-specific CD8+ T Cells⁶
 - Tadalafil also has been shown to reverse tumor-specific immune suppression in HNSCC⁷
- Opportunities for improved techniques to characterize the tumor immune microenvironmental interactions should be explored to develop novel therapies
 - Immunohistochemistry uses only 1-2 antibodies in one image, and flow cytometry processing loses important tumor architecture information

Our objectives were to:

- Validate utility of Co-Detection by indExing (CODEX®; Akoya BioSciences) in HNSCC by comparing results of immune cell population concentrations acquired from flow cytometry compared to data from CODEX® analysis on harvested HNSCC tumor samples.
- Use spatial analysis to assess the topographical associations between PD-1+ and PDL-1+ cell populations within the HNSCC tumor immune microenvironment.

Methodology

- Performed an IRB-approved prospective clinical study of patients (N=10) with Stage II-IV surgically-resectable primary or recurrent HNSCC with or without prior radiation at a tertiary care NCI-designated Cancer Center
- Tumor was harvested at time of surgery of at least 2 cubic cm. No tumor samples could contain peripheral aspect at tumor-normal junction as this would affect oncologic margin analysis
- CODEX®**
 - Half of the tumor sample was kept intact and immediately flash frozen in OCT compound, & stored in -80 freezer. Samples were later sliced by a pathologist onto poly-L-lysine coated tissue slides
 - Tissue slides for each tumor sample were stained with CODEX®-conjugated antibodies, washed, tagged with a fluoroscopic oligonucleotide barcode, and run through the CODEX® machine for imaging of three markers at a time in 10 cycles per manufacturer protocol
 - Data acquired from images were analyzed at the single cell level and gated for leukocytic cell populations to compare cell percentages to data acquired from flow cytometry on the same tissue
 - Images acquired were run through Multiplex Analysis Viewer programming and color-coded by marker (Figure 1). Up to 5 colors used at one time per image given that the human eye can only distinguish about 5 colors in one image distinctly (Figure 2)
- Flow Cytometry**
 - The other half of tumor was processed for flow cytometry, which involved breakdown with collagenase incubations and staining with same antibodies chosen for CODEX® panel for FACS analysis to determine percentages of leukocytic cells based on markers of expression
 - Paired T-test** performed to compare cell percentages between flow cytometry- and CODEX®-acquired data
 - Spatial analyses** performed between PDL-1+ tumor, PDL-1+ leukocytes, and exhausted (PD-1+) CD8 and CD4 T cells on tumor samples

Results

1. Demographics, subsite, staging, and pathologic data of the 10 included patient tumor samples (Table 1).

- 80% Male, average age of 68 years, 60% positive smoking history, 50% recurrent, 50% with prior radiation, 80% were of oral cavity primary, no patients with HPV-related disease, 50% with perineural invasion, only 1 sample with lymphovascular invasion

2. Simple gating strategies using the 30 antibodies stained on the tissue allow for specific identification of leukocyte cell populations, and Tsne plots indicate predictable lack of overlap between distinct subsets (Figures 1, 2, 3)

3. Flow Cytometry vs CODEX Technology for Detection of Percentage of Leukocytes (Figure 4)

- Flow cytometry detects significantly less CD45+ leukocytes ($p=0.0026$), CD15+ neutrophils and granulocytic MDSCs ($p=0.006$), and CD14+ monocytes and monocytic MDSCs ($p=0.017$)
- Flow cytometry detects significantly more CD3+ lymphocytes ($p=0.00418$), particularly significantly more CD8+ cytotoxic T cells ($p=0.027$) and activated cytotoxic T cells ($p=0.046$)

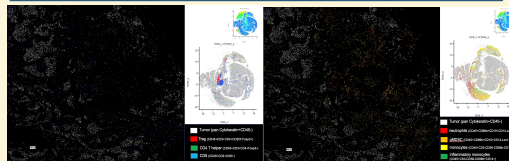
4. Spatial analyses performed between PDL-1+ tumor and leukocytes and the exhausted (PD-1+) CD4 and CD8 T cells (Figure 5)

- PDL-1+ tumor completely excludes leukocytes and exhausted CD8 ($p=0.009$) and CD4 ($p<0.001$) T cells

Table 1: Characteristics of HNSCC Tumor Samples

Patient Sample	Site	Pathologic Stage AJCC 8	Recurrence?	Largest size of tumor	PIH	VI	DCI	Previous radiation	Age	Gender	HPV-related
1	Oral Cavity (Oropharynx)	pT2N0M0a	No	3.5 cm	Present	No	1.5 cm	No	56	M	No
2	Hypopharynx	pT2N0M0a	Yes	1.1 cm	Negative	No	0.2 cm	Yes	62	M	No
3	Oral Cavity (Tongue)	pT2N0M0a	No	3 cm	Present	Yes	1.8 cm	No	77	M	No
4	Oral Cavity (Hard palate)	pT2N0M0a	Yes	2.3 cm	Negative	No	0.6 cm	Yes	75	F	No
5	Oral Cavity (Tongue)	pT2N0M0	No	3.9 cm	Present	No	3.7 cm	No	61	F	No
6	Oral Cavity (Tongue, PSM)	pT2N0M0	Yes	3.0 cm	Negative	No	0.6 cm	Yes	64	M	No
7	Oropharyngeal	pT2N0M0a	Second Primary	1.5 cm	Present	No	N/A	Yes	64	M	No
8	Oral Cavity (Mandibular Alveolar Ridge)	pT2N0M0a	No	3.0 cm	Negative	No	1.1 cm	No	78	M	No
9	Oral Cavity	pT2N0M0a	Yes	5.8 cm	Negative	No	0.5 cm	No	72	M	Yes
10	Oral Cavity (Tongue)	pT2N0M0a	Yes	2.4 cm	Positive	No	1.3 cm	Yes	71	M	No

Figure 1: Tsne Plots Help Validate CODEX® by Confirming Expected HNSCC Leukocyte Subset Expression Patterns



Example from single patient tumor sample image after gating strategies employed to select out specific leukocyte populations (listed in color-coded key bottom right) with associated Tsne plots. Tsne plots show that gated subsets are distinct populations from each other, without overlap between T cells on the left image and monocytes and granulocytes on the right image.

Figure 2: CODEX® Allows for the High-Resolution Detection of 30 Markers in the Tumor Microenvironment

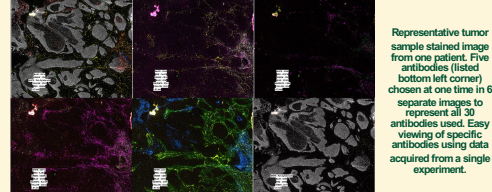


Figure 3: Easily Visualize Cellular Interactions by Assigning 4-5 Colors

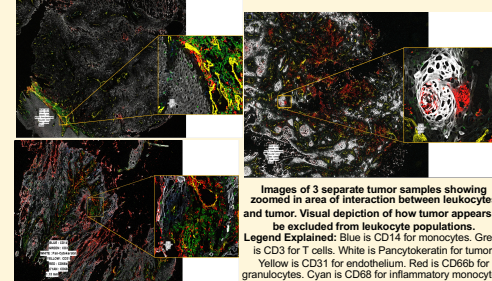
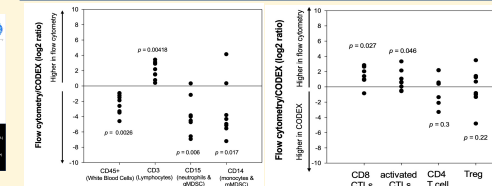
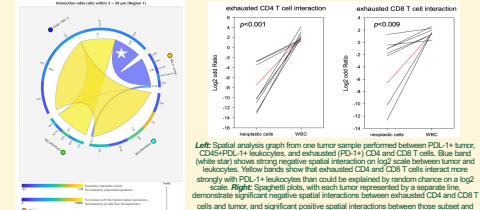


Figure 4: CODEX® Outperforms Flow Cytometry for the Quantification of Tumor-Infiltrating Leukocytes & Granulocytes



Dot Plots with each tumor sample represented by a single dot, and paired T-test performed comparing percentage of cells by marker after performing gating for flow cytometry and CODEX.

Figure 5: CODEX® Allows for the Evaluation of the Spatial Interaction Between Cell Subsets



Conclusions

- CODEX® may be a valid tool for analysis of key leukocyte populations within HNSCC tumor microenvironment**
 - Expected division of leukocyte cell populations supported by Tsne plots
 - Includes tumor cells for improved analysis of tumoral interactions than what can be obtained from flow cytometry, and ability to easily use >30 antibodies in one experiment allows for more complex analyses of spatial interactions than what can be obtained from immunohistochemistry
- Flow cytometry may underestimate leukocytes, particularly granulocytes and myeloid cells**
 - We posit exposure to collagenase during processing of specimen for flow cytometry degranulates these cells thus they get lost in spectrometry
- Flow cytometry may overestimate cytotoxic T cells**
- CODEX® allows for specific spatial analyses in the HNSCC tumor through preservation of tissue architecture**
- Future studies can be performed using CODEX® to advance immunotherapies and develop new stratification tools in HNSCC**
 - Topographical patterns may allow for a new stratification tool for predicting responses to immunotherapy agents in clinical trials
 - Novel spatial interaction data can be used prospectively to develop a stratification tool to predict risk of recurrence or survival
 - Identify changes in pre-treatment biopsy spatial interactions to post-treatment biopsy interactions with immunotherapy treatments and correlate with treatment success
 - Does checkpoint inhibitor therapy change the PDL-1+ tumor exclusion properties identified in our study to enhance effector T cell anti-tumor activity?
 - Correlate spatial interaction data with pathologic features and history of radiation

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