Using a Multiplexed Immunofluorescence Approach to Compare Immune Cell Populations in Subtypes on Non-Small Cell Lung Cancer

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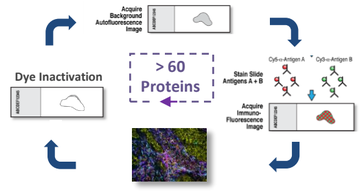
Background and Results

Background: Non-small cell lung cancer (NSCLC) accounts for approximately 80-85% of all lung cancer cases, and is characterized by a poor response to chemotherapy and a low survival rate. NSCLC is a very heterogeneous disease with the two most common types being adenocarcinoma (ADCA) accounting for about 70% of all NSCLC cases, and squamous cell carcinoma (SCC) accounting for about 20% of all cases.
Treatment targeting the immune checkpoint inhibitor pathway PD-1/PD-L1 has been found to be effective against NSCLC with manageable side effects, but with only 20-25% of patients showing a positive response there is an urgent need for additional immunotherapy options for this group of patients.

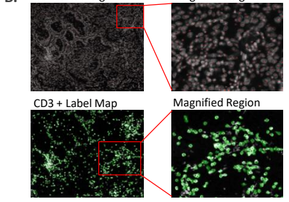
Methods: We used MultiOmyx™, an immunofluorescence (IF) multiplexing assay that utilize a pair of directly conjugated Cyanine dye-labeled (Cy3, Cy5) antibodies per round of staining. Using a 16-marker panel we have analyzed the proportion of B cells, T cell subtypes, M1/M2-type tumor-associated macrophages, as well as the expression of not only PD-1 and PD-L1, but also of more novel immunotherapy targets LAG-3 and TIM-3 in 19 FFPE samples from patients with NSCLC (10 ADCA and 9 SCC).

Results: We observed a higher density of the immunosuppressive cell types Tregs and M2 type TAMs in SCC versus ADCA tumors. LAG-3 was found to be expressed mainly on T cytotoxic cells in both subtypes, but the overall density of LAG-3 was 59% higher in SCC. TIM-3, primarily found expressed on TAMs had a 168% higher density in SCC, while the density of PD-L1 was 33% lower in SCC compared to. When analyzing the proportion of T cytotoxic tumor-infiltration, we observed an apparent synergy between LAG-3 and PD-1 co-expression in both ADCA and SCC, suggesting a therapeutic benefit of dual checkpoint blockade of LAG-3 and PD-1 in NSCLC.

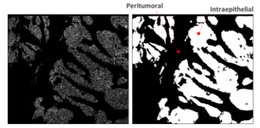
MultiOmyx™ Hyperplexed IF Assay Workflow



MultiOmyx™ Deep Learning Cell Classification



MultiOmyx™ Tumor Segmentation Algorithms



NeoVUE™ Image Visualization Tool

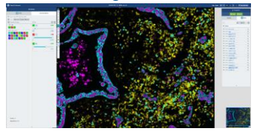


Figure 1. MultiOmyx™ Assay Workflow. Slides were prepared and stained using MultiOmyx™ multiplexing IF staining protocol. **A.** For each round of staining, conjugated fluorescent antibodies were applied to the slide, followed by image acquisition of stained slides. The dye was erased, enabling a subsequent round of staining with another pair of fluorescent antibodies. **B.** Proprietary cell segmentation algorithms generate unique IDs for every cell allowing them to be tracked through multiple rounds of staining. Deep learning based cell classification algorithms identify positive cells for each biomarker which are visualized using via Label masks shown here for CD3. **C.** A tumor marker such as pan-cytokeratin is used to generate a tumor "mask" in order to classify all cells as intraepithelial or Peritumoral. **D.** The image and data visualization software NeoVUE™ was used to create all overlay images for this study.

16-Marker Panel		
Round	**Cy3**	**Cy5**
1	CD15	PD-L1
2	PanCK	ICOS
3	CD11b	LAG-3
4	CD3	OX40
5	CD8	TIM-3
6	CD4	FoxP3
7	CD20	CD163
8	CD68	PD-1

Table 1. Antibody Staining Sequence for MultiOmyx multiplexing staining.

Nomenclature	Tumor Tissue Phenotype
T helper cells	CD3+CD4+ (+/- PD-1/LAG-3/TIM-3)
T regulatory cells	CD3+CD4+FoxP3+ (+/- PD-1/LAG-3/TIM-3)
T cytotoxic cells	CD3+CD8+ (+/- PD-1/LAG-3/TIM-3)
TAMs	CD3-CD68+ (+/- TIM-3 or PD-L1)
M2 TAMs	CD3-CD68+HLA-DR-CD163+
PanCK	Tumor cells (+/- PD-L1)

Table 2. Phenotyping of human tumor-associated lymphocytes and myeloid cells. Cell surface markers associated with cell subsets analyzed in the tumor samples. TAM: tumor-associated macrophage. PanCK: pan cytokeratin.

Immune Cell Phenotypes in ADCA versus SCC subtypes of NSCLC

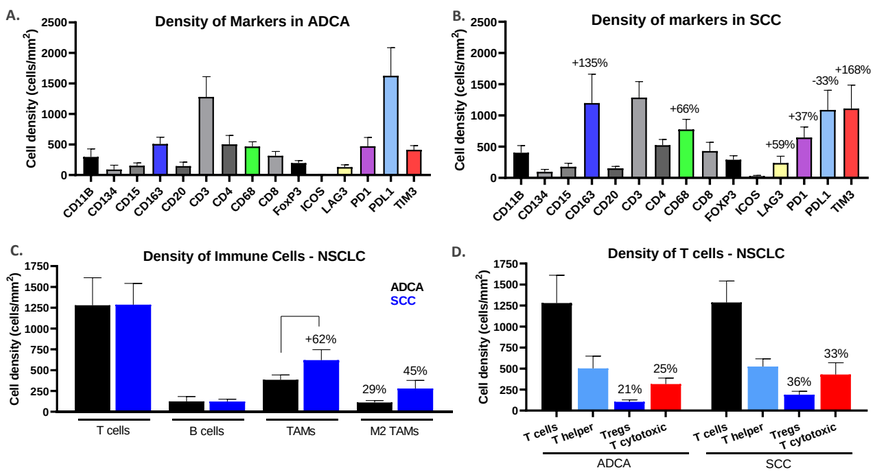


Figure 2. Immuno-profiling of FFPE NSCLC samples using MultiOmyx. Densities of all 15 markers were calculated following MultiOmyx multiplex staining protocol, using deep learning based cell classification algorithms for **A**. ADCA and **B**. SCC samples. **C**. when comparing the densities of T cells (CD3+), B cells (CD3-CD20+), TAMs (CD3-CD68+), and M2-type TAMS (CD3-CD68+CD163+) we observed and increase in both TAMs overall as well as the proportion of M2 TAMS in SCC compared to ADCA. **D**. when comparing the proportions of T cell subsets we observed and increase in both T regulatory cells (CD3+CD4+FoxP3+), and T cytotoxic cells (CD3+CD8+) in SCC compared to adenocarcinoma.

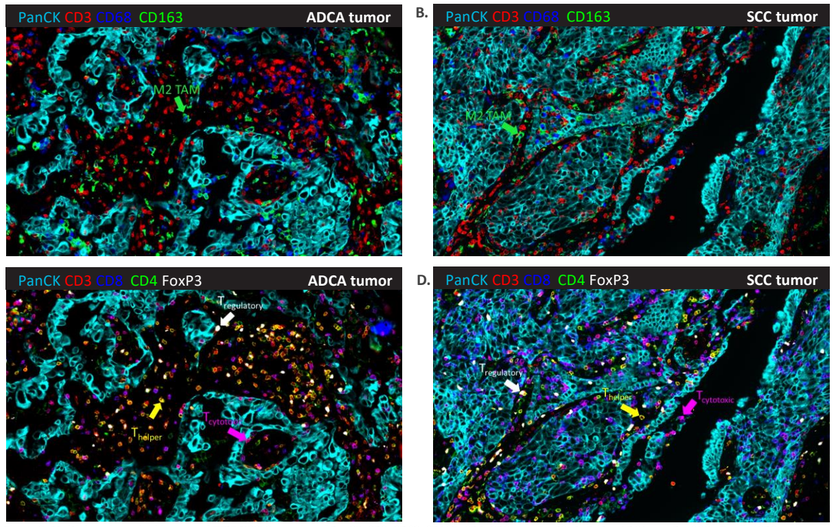


Figure 3. Multiplexed overlaid images of FFPE NSCLC samples of PanCK, CD3, CD68, CD163, CD4, CD8, and FoxP3. **A+B (A** = ADCA and **B** = SCC). **T cells** (CD3+) are red, and **M2-TAMs** are green and blue (CD68+CD163+). **C+D (C** = ADCA, and **D** = SCC). **T cells** (CD3+) are red, **T helper** cells (CD3+CD4+) are yellow, **T cytotoxic** cells (CD3+CD8+) are magenta, and T regulatory cells (CD3+CD4+FoxP3+) are white and yellow.

LAG-3 and TIM-3 Immune Cell Expression, and Co-Expression with PD-1

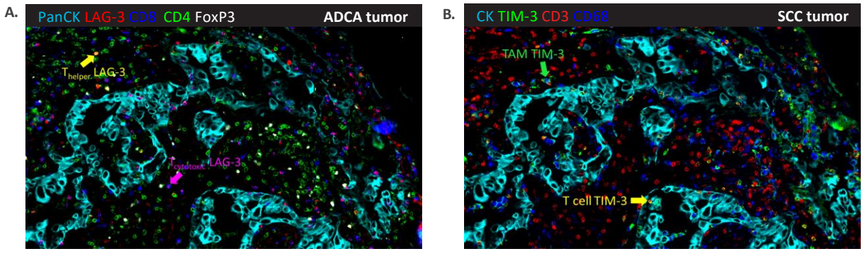
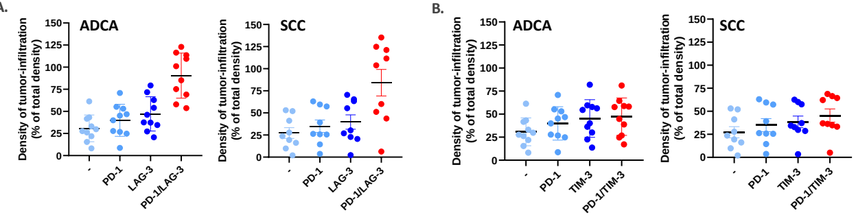


Figure 4. Multiplexed overlaid images of PanCK, CD3, CD4, FoxP3, CD68, LAG-3, and TIM-3. **A** (ADCA) **T helper** cells expressing LAG-3 **(**CD4+LAG-3+) are yellow,while **T cytotoxic** cells (CD8+LAG-3+) are magenta. **B** (SCC) **T cells** expressing TIM-3 **(**CD3+TIM-3+) are yellow, while **TAMs** expressing (CD68+TIM-3+) TIM-3 are green and blue .

Higher Tumor-Infiltration of T cytotoxic Cells when LAG-3 and PD-1 are Co-Expressed



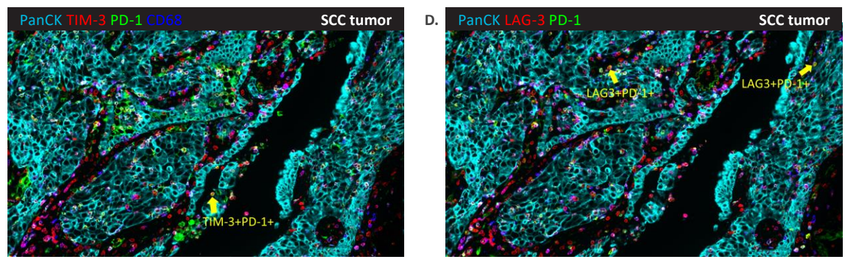


Figure 5. Tumor infiltration analysis using tumor masks generated as described in figure 1 C, classifying all T cytotoxic cells as either peritumoral (non-infiltrating) or intra-epithelial (infiltrating). **A**. More T cytotoxic cells infiltrate the tumor are when co-expressing LAG-3 and PD-1, than cells expressing LAG-3 or PD-1 alone, in both ADCD and SCC. **B**. This apparent synergy is not observed for TIM-3 and PD-1 co-expression. **C.** Cells co-expressing TIM-3 and PD-1 are yellow. **D**. Cells co-expressing LAG-3 and PD-1 are yellow.

Conclusion

In this study, utilizing MultiOmyx™ technology, a platform offered exclusively by NeoGenomics Laboratories, protein expression in 19 patients with lung cancer were analyzed for a comparison of the NSCLC subtypes adenocarcinoma (ADCA) and squamous cell carcinoma (SCC).

- Utilizing a panel of 16 antibody markers we quantified the number of T cells, B cells, TAMs, and M2-type TAMs and observed an increase in the proportion of the immunosuppressive cell types T regulatory cells, and M2 TAMS in SCC versus ADCA.
- Additionally, SCC samples had a higher density of both LAG-3 and TIM-3. In both subtypes LAG-3 was found primarily expressed on T cytotoxic cells, while TIM-3 was found primarily on TAMs, followed by an equal distribution on helper and cytotoxic T cells.
- When analyzing T cytotoxic tumor-infiltration we found that in both ADCD and SCC, cells co-expressing LAG-3 and PD-1 infiltrated the tumor area more so than cells expressing LAG-3 or PD-1 alone. This apparent synergy between LAG-3 and PD-1 was not observed for TIM-3 and PD-1.

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