LB-C18

An Integrated Multiplexing Approach for the Immunoprofiling of the Tumor Microenvironment of Ovarian Granulosa Cell Tumors

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Background and Results

Background: Granulosa cell tumors (GCTs) are rare tumor in ovaries accounting for 2-5% of all ovarian cancers. GCT malignancies are often low-grade with a five-year survival rate up to 90%, however a clinical characteristic of these tumors is a tendency for late recurrence and a high recurrent rate is the most critical factor for GCT death. As GCTs are rare tumors and tissue availability is very limited, we used a dual multiplexing approach in order to maximize the data output from a total of 14 FFPE rare GCT tumor samples. Gene and protein levels in these 14 GCT samples were compared to levels in 5 high-grade serous ovarian cancer (HGSOC) FFPE samples.

Methods: For protein multiplexing we have used MultiOmyx™, an immunofluorescence (IF) multiplexing assay utilizing a pair of directly conjugated Cyanine dye-labeled (Cy3, Cy5) antibodies per round of staining. Each round of staining is imaged and followed by dye inactivation, and deep learning based cell classification algorithms identify positive cells for each. Gene expression analysis was done using the Nanostring PanCancer Immune 770 gene panel assay. RNA was extracted from the adjacent 10 µm section and then proceeded with hybridization, purification and immobilization and count based on manufacturer's protocol.

Results: On protein level we confirmed previous findings that ovarian tumors are so-called "cold" tumors, with a very low density of T cell infiltration which is even further reduced in GCT samples compared to HGSOC samples. This is also reflected by a significant decrease in the gene score for cytotoxic cells. When analyzing tumor markers S100, vimentin, and pan-cytokeratin in GCT samples we observed an almost complete loss of cytokeratin, but increases in vimentin and S100 (protein level), and a highly significant mRNA decrease in MUC1 which codes for EMA, a negative marker for adult GCT.

On both mRNA and protein level we found a reduction in macrophages in GCT samples, and on protein level we also observed a reduction in proliferation marker Ki67. Density of angiogenic vessels in the GCT microenvironment was increased, possibly linked to a highly significant increase in NOS2A mRNA which codes for the protein nitric oxide synthase (NOS2), a known modulator of angiogenesis.

Multiplexing Setup – MultiOmyx™ (protein) & NanoString nCounter® (mRNA)

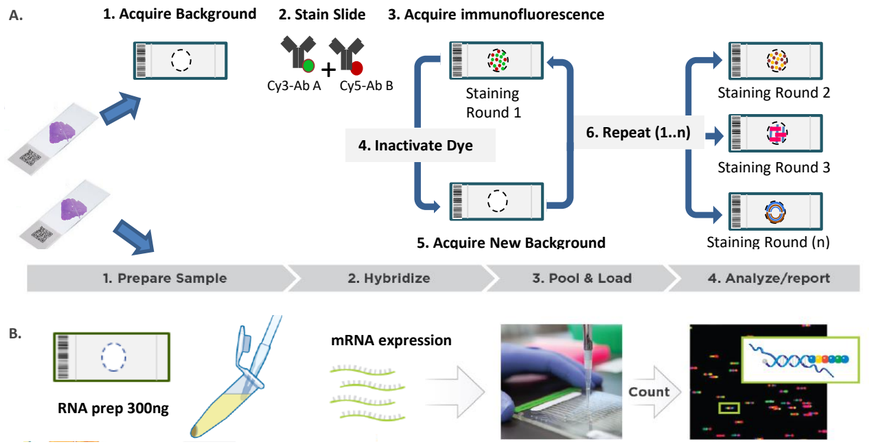


Figure 1. Multiplexing Assay Workflow. Two adjacent sections were cut from each FFPE tumor sample. **A. MultiOmyx multiplexing IF staining protocol**. For each round of staining, conjugated fluorescent antibodies were applied to a 4 µm section, followed by image acquisition of stained slides. The dye was erased, enabling a subsequent round of staining with another pair of fluorescent antibodies. Proprietary cell segmentation algorithms generate unique IDs for every cell allowing them to be tracked through multiple rounds of staining. **B. Nanostring nCounter assay**. RNA was extracted from the adjacent 10 µm section and then proceeded with hybridization, purification and immobilization and count based on manufacturer's protocol.

Panel Specifications

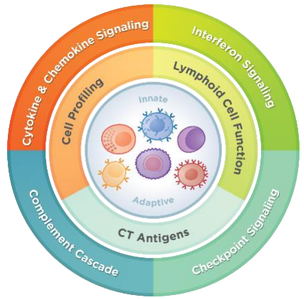
16-Marker Panel		
#	Cy3	Cy5
1	CTLA-4	CD34
2	PanCK	CD163
3	CD4	PD-1
4	CD3	PD-L1
5	CD8	FoxP3
6	CD20	HLA-DR
7	Ki67	CD68
8	Vimentin	S100

Co-expression	Phenotypes
CD3+CD4+	T helper
CD3+CD4+FoxP3+	T regulatory
CD3+CD4+PD1+	T helper PD-1
CD3+CD4+FoxP3+PD1+	T regulatory PD-1
CD3+CD4+CTLA4+	T helper CTLA-4
CD3+CD4+FoxP3+CTLA4+	Treg CTLA-4
CD3+CD8+	T cytotoxic (CTL)
CD3+CD8+PD1+	CTL PD-1
CD3+CD8+CTLA-4+	CTL CTLA-4
CD3-CD20+	B cells
CD3-CD68+	TAM
CD3-CD68+Ki67+	Proliferating TAM
CD3-CD68+HLADR+CD163-	M1 TAM
CD3-CD68+HLADR-CD163+	M2 TAM
PanCK+Ki67+	Tumor proliferation

Table 1. Antibody Staining Sequence for MultiOmyx multiplexing staining. 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.

PanCancer Immune Profiling Panel :

- 770 genes from 24 different immune cell types, common checkpoint inhibitors, and CT antigens.
- Assesses mechanistic pathway activity.
- Identifies TILs in the tumor microenvironment.
- 40 reference genes.



NanoString nCounter data – Cell Type Profiling & Differential Expression

Reduced expression of genes for cytotoxic cells and TAMs in GCT samples

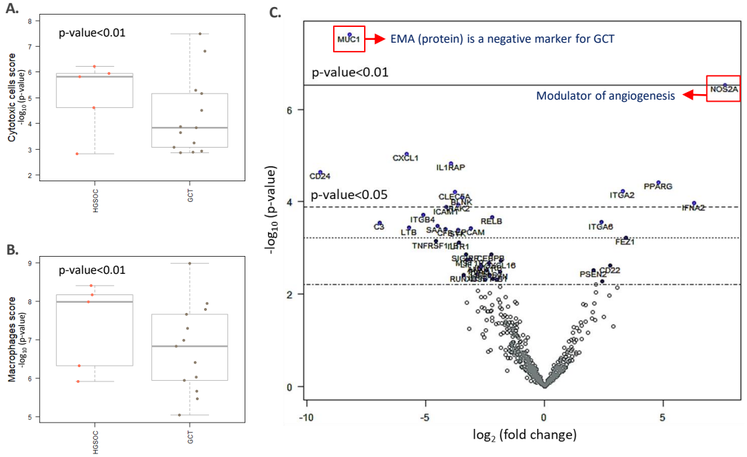


Figure 2. Nanostring Cell Type Profiling & Differential Expression. A+B the gene scores for cytotoxic cells (PRF1, GNLY, GZMA) and macrophages (CD68, CD163, CD84) were significantly reduced in GCT samples compared to HGSOC control samples. **C Volcano plot** displaying each gene's -log10(p-value) and log2 fold change with the selected covariate. Highly statistically significant genes fall at the top of the plot above the horizontal lines, and highly differentially expressed genes fall to either side. Horizontal lines indicate various False Discovery Rate (FDR) thresholds or p-value thresholds if there is no adjustment to the p-values.

MultiOmyx Overlay Images – Tumor Markers, TILs, TAMs, & Vessels

Decrease in cytokeratin, CTLs, TAMs, Ki67 in GCT samples, but increase in S100, vimentin & CD34.

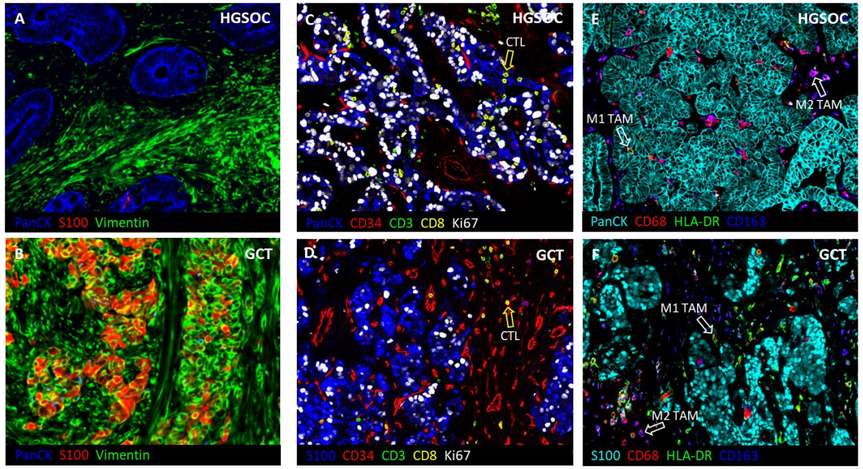
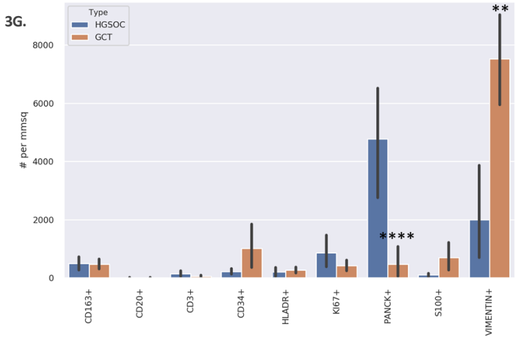
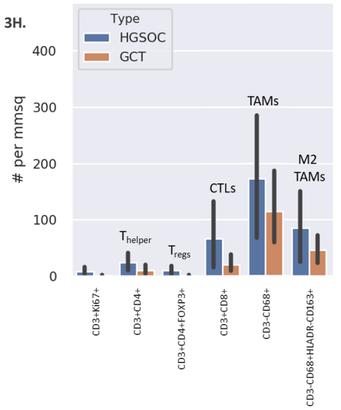


Figure 3. MultiOmyx Multiplexed Images & Quantitation. A-F. Multiplexed overlay images of PanCK, S100, vimentin, CD34, CD3, CD68, Ki67, CD68, HLA-DR, and CD163. **A+C+E** = HGSOC and **B+D+F** = GCT. **G+F**. Quantitation of protein density (cell #/mm^2) of single markers (**G**), and co-expressions for T helper cells (CD3+CD4+), T regulatory cells (CD3+CD4+FoxP3+), cytotoxic lymphocytes/CTLs (CD3+CD8+), tumor-associated macrophages/TAMs (CD3-CD68+), and M2-type TAMs (CD3-CD68+HLADR-CD163+).

MultiOmyx Data – Protein Density Quantitation





Key Findings

- We have used a dual multiplexing approach to immunoprofile the tumor microenvironment of rare ovarian Granulosa Cell Tumor FFPE samples on both mRNA level (Nanostring nCounter assay), and protein level (MultiOmyx analysis).
- Gene signatures and protein levels for T cytotoxic lymphocytes and tumor-associated macrophages are reduced in GCTs compared to control HGSOC tumors.
- Angiogenic vessel protein density is increased in GCTs, possibly linked to a highly significant over-expression of the gene for NOS2 which is a modulator of angiogenesis.

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