#5135 Detection of IFNγ induced PD-L1 expression by combined in situ RNA analysis and protein profiling from a single FFPE slide

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Background

PD1 ligands (PD-L1) are often upregulated on the cell surface of many different tumors. The primary role of PD-L1 in cancer is to inhibit T-cell mediated immune response. Two general mechanisms for PD-L1 expression on tumor cells have been proposed. Innate immune resistance, in which PD-L1 expression is induced by the constitutive oncogenic signaling, and adaptive immune resistance, in which PD-L1 expression is induced by T-cells releasing interferon-γ (IFNγ) and activating the STAT signaling pathway. In order to differentiate between these two mechanisms, IFNγ mRNA expression is measured as an effective alternative to detecting IFNγ protein. Detection of cytokines by IHC is challenging as secreted proteins are widely diffused and the associated staining pattern appears to lack cellular specificity. RNAscope RNA in situ hybridization (ISH) assay is utilized to measure IFNγ mRNA expression, and MultiOmyx™ multiplexed immunofluorescence (IF) assay (demonstrated to stain up to 60 protein biomarkers) is utilized to measure CD3, CD4, CD8, FOXP3, CD163, TIM3, LAG3, PD1, and PD-L1 protein expression. In this study, combined MultiOmyx multiplexing IF and RNAscope ISH assay, enabled identification of individual cells with characteristic mRNA and protein expression profile in lung, breast, melanoma, colorectal, esophageal, and prostate cancer samples.

Overview of Combined RNAScope ISH and MultiOmyx Multiplexing IF Workflow

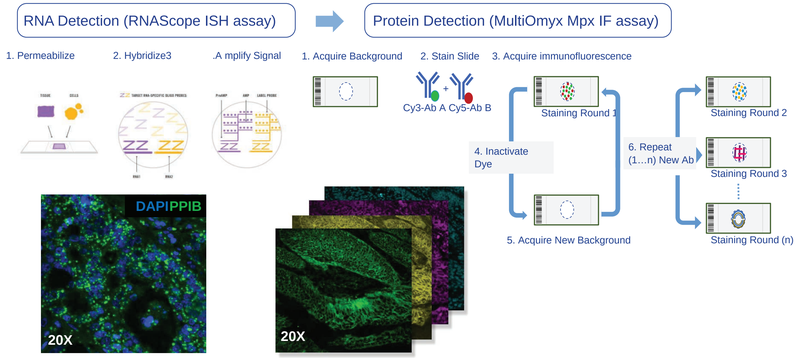


Figure 1. MultiOmyx multi "Omic" scheme for RNA analysis and protein profiling from a single tissue section. Slides were cleared per MultiOmyx standard slide preparation procedures and then processed through pre-treatment, hybridization and signal amplification steps based on RNAScope manufacturer's protocol. After RNA signals were captured, the same slide was processed using MultiOmyx multiplexing IF staining protocol. For each round of staining, conjugated fluorescent antibodies were applied to the slide, followed by slide stained imaging. The dye was chemically inactivated, enabling a second round of staining with another pair of fluorescent antibodies. The process was performed multiple times from a single slide.

Conclusion

Combined MultiOmyx IF and RNAscope assays enabled direct correlation between IFNγ mRNA expression to PD-L1 protein expression, in order to differentiate PD-L1 expression induced in response to inflammatory signals, from PD-L1 expression induced by constitutive oncogenic signaling. Lung sample B illustrate a possible adaptive immune resistance phenotype in which PD-L1 expression is induced in tumor cells in response to IFNγ. High IFNγ expression is observed in tumor/CK+ cells and in CD8+ T cytotoxic cells, indicated by cyan and white arrows in figure 3A. It is accompanied by high tumor infiltrating immune cells consisting of Tcytotoxic, Thelper, Tregulatory, and macrophages. In lung sample A, PD-L1 is expressed in tumor/CK+ cells similar to sample B, but PD-L1 expression is not induced by IFNγ expression. Lung sample A illustrates a possible innate immune resistance phenotype in which PD-L1 expression is induced by an oncogenic pathway, independent of inflammatory signals in the tumor microenvironment. The MultiOmyx assay enables differentiation of PD-L1 innate immune resistance from adaptive immune resistance by integrating "multi-omic" RNA analysis and protein profiling from a single FFPE slide.

Biomarker Staining Comparison Between Individual MultiOmyx Assay to Combined RNAScope & MultiOmyx assay

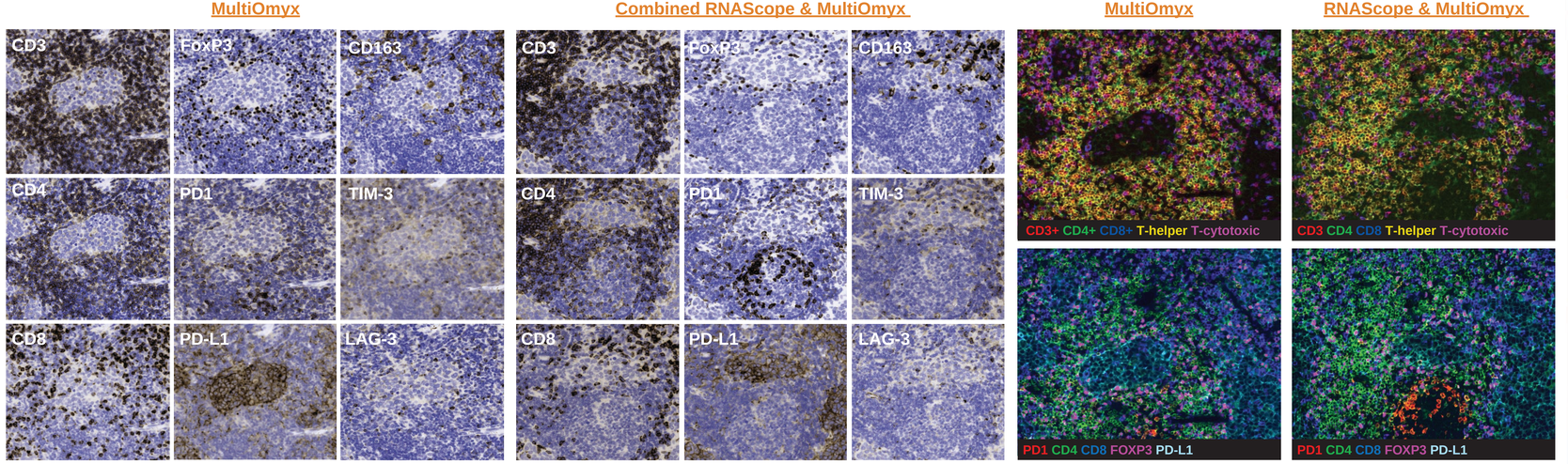
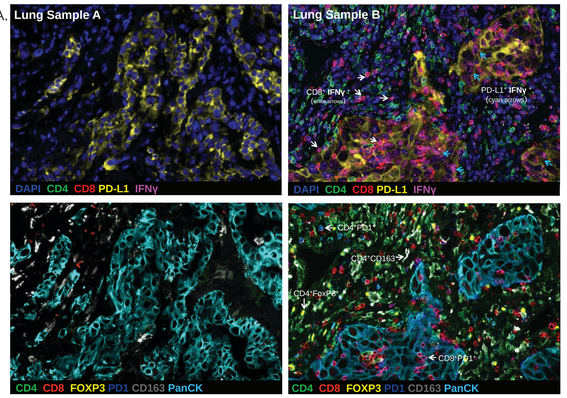


Figure 2. Comparison of biomarker staining on RNAScope & MultiOmyx IF processed slides vs MultiOmyx IF only (20x). The left panel displayed representative biomarker staining shown as 'molecular' DAB or mDAB images from MultiOmyx IF staining processed slide. The middle panel displayed slides processed through combined RNAScope and MultiOmyx staining. All nine biomarkers showed robust specific staining. The right panel displayed color overlaid images of multiplexed staining as two sets of images to improve visualization.

Integrated RNA Analysis and Protein Profiling

A.



B.

Co-expression	# of cells	% of cells	Co-expression	# of cells	% of cells
CD3+CD4+	< 10	-	CD3+CD8+	< 10	-
CD3+CD4+PD1+	0	0	CD3+CD8+PD1+	0	0
CD3+CD4+TIM3+	< 10	-	CD3+CD8+TIM3+	0	0
CD3+CD4+PD1+TIM3+	0	0	CD3+CD8+PD1+TIM 3+	0	0
CD3+CD4+FoxP3+	0	0	CD3-CD163+	97	14.5
CD3+CD4+ FOXP3+PD1+	0	0	CD3-CD163+ PDL1+	< 10	-
CD3+CD4+ FOXP3+TIM3+	0	0	PanCK+PDL1+	148	22.2
DAPI (# of total cells)	667	-			

Co-expression of immune markers in lung sample A

Co-expression	# of cells	% of cells	Co-expression	# of cells	% of cells
CD3+CD4+	219	14.3	CD3+CD8+	206	13.4
CD3+CD4+PD1+	53	3.5	CD3+CD8+PD1+	40	2.6
CD3+CD4+TIM3+	33	2.2	CD3+CD8+TIM3+	43	2.8
CD3+CD4+ PD1+TIM3+	11	0.7	CD3+CD8+ PD1+TIM3+	18	1.2
CD3+CD4+FoxP3+	41	2.7	CD3-CD163+	379	24.7
CD3+CD4+ FOXP3+PD1+	< 10	-	CD3-CD163+ PDL1+	19	1.2
CD3+CD4+ FOXP3+TIM3+	< 10	-	PanCK+PDL1+	158	10.3
DAPI (# of total cells)	1533	-			

Co-expression of immune markers in lung sample B

C. Spatial analysis of T-cells & Mφ

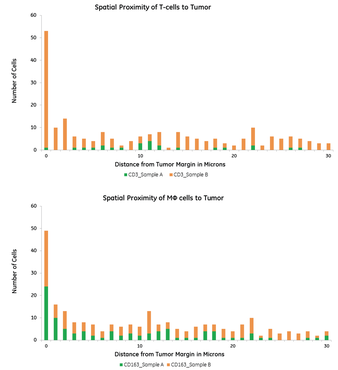


Figure 3. Characterization of IFNγ mRNA expression and protein profiling at single cell level by combined RNAScope ISH and MultiOmyx multiplexing IF assay. (A) Color overlaid images of the punctate IFNγ mRNA signals detected by RNAScope (in magenta) and PD-L1, CD4, CD8 MultiOmyx IF staining (in yellow, green, red) and DAPI (in blue) on 2 FFPE lung cancer samples with high PD-L1 expression. (B) Co-expression analysis of multiple immune markers utilizing MultiOmyx proprietary cell classification algorithm. For each phenotype, the number and percentage of cells are calculated. (C) Spatial analysis calculating the distance of T cells and macrophages in proximity to the tumor margin.