Co-detection of a tumor-infiltrating lymphocyte immunofluorescence panel and cytokine RNA in-situ hybridization markers in non-small cell lung cancer tumor microenvironment using combined MultiOmyx™ and RNAscope® platforms

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#2681

Introduction

Co-detection of RNA and protein can greatly expand the data output from a single specimen, providing critical information such as the source of secreted proteins (e.g. cytokines) or cell type specific transcript levels. MultiOmyx is a proprietary immunofluorescence (IF) platform for the visualization and characterization of up to 60 protein biomarkers in a single formalin-fixed paraffin-embedded (FFPE) section. RNAscope Multiplex is a highly sensitive fluorescent in-situ hybridization (ISH) assay that can detect up to 3 RNA markers in a single FFPE section. Combination of MultiOmyx IF with RNAscope Multiplex ISH would therefore provide a novel and powerful platform to co-detect multiple RNA and protein markers in a single slide/sample. However, the RNAscope Multiplex assay includes a protease pretreatment step, which may compromise downstream antibody-antigen interaction and thereby the IF signal. This study is a validation of the sensitivity, specificity, reproducibility, and repeatability of an integrated MultiOmyx IF and RNAscope ISH assay.

Depending on the context, cytokines interleukin-10 (IL10) and interferon gamma (IFNγ) have both been shown to either induce immunosuppression and favor tumor growth or promote an anti-tumor response. The mechanisms and cues determining the pro- or anti-tumor activity of IL10 and IFNγ are still poorly understood. Spatiotemporal characterization of IFNG and IL10 is therefore critical to help define the dynamic relationship of cytokines and the immune system within the tumor microenvironment. Therefore, for validation of the integrated MultiOmyx-RNAscope platform, RNA ISH markers for IL10 and IFNγ were combined with the MultiOmyx 12 marker tumor infiltrating lymphocyte (TIL) panel (CD3, CD4, CD8, CD20, CD68, CD56, CD45RO, PD-1, PD-L1, CTLA4, FOXP3, and tumor marker PanCK) on FFPE human NSCLC samples. This combined MultiOmyx-RNAscope workflow was performed for three individual runs using triplicate NSCLC samples for each run. Intensity and cell classification for each ISH and TIL marker was quantified using the proprietary MultiOmyx Analytics pipeline.

The results demonstrate that the integrated assay maintains sensitivity and specificity of the TIL IF markers in the integrated workflow when benchmarked to an IF alone workflow. Furthermore, these results can be used to characterize expression of IL10 and IFNγ within immune cell subsets represented by the TIL IF panel (e.g. T cells, B cells, macrophages). This integrated approach can be used to spatially correlate the distribution of cytokine and immune cell expression within the tumor microenvironment. Therefore, the RNAscope-MultiOmyx IF assay provides a robust and powerful platform to simultaneously co-detect RNA with protein IF in a single specimen.

Overview of Combined RNAscope ISH and MultiOmyx IF Workflow

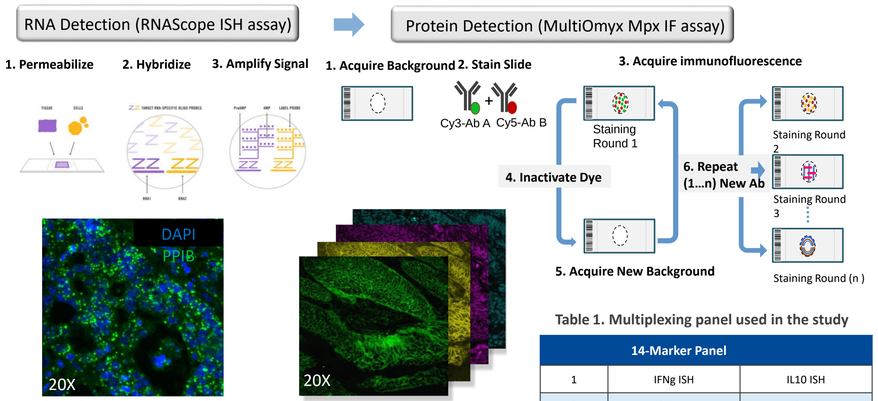


Figure 1. MultiOmyx multi "Omic" scheme for RNA analysis and protein profiling from a single tissue section. Slides were cleared per MultiOmyx standard slide preparation procedures and then processed through pre-treatment, hybridization and signal amplification steps based on RNAscope manufacturer's 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.**

Table 1. Multiplexing panel used in the study

14-Marker Panel		
1	IFNg ISH	IL10 ISH
2	CTLA-4	CD56
3	PanCK	CD45RO
4	CD8	FOXP3
5	CD3	PD-L1
6	CD4	PD-1
7	CD20	CD68

Staining Concordance between the Integrated Assay and IF Assay

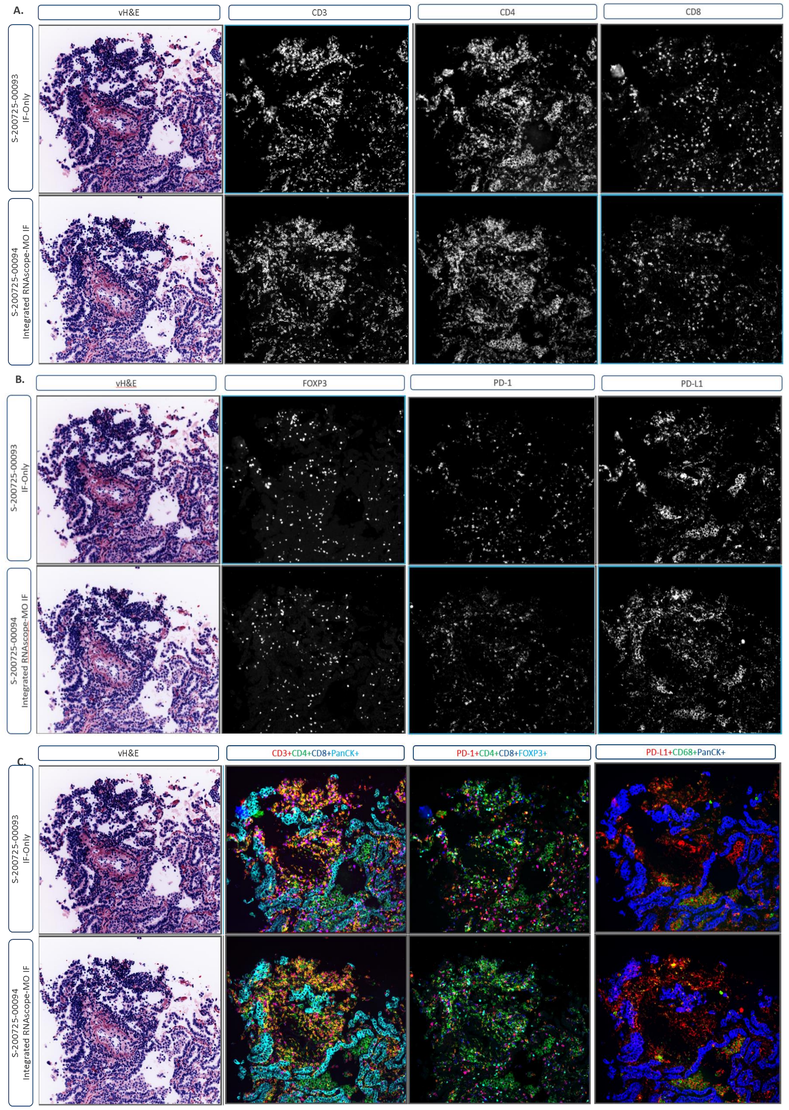


Figure 2. Comparison of biomarker staining in integrated assay vs IF assay in NSCLC sample. A. T cell marker staining (CD3, CD4 & CD8) from one representative region of interest (ROI). B. Representative immune modulator staining (FOXP3, PD-1, PD-L1) from the same set of ROIs. In Figure A and B, the top row is the biomarker staining from IF assay alone and the bottom row is the representative staining from the integrated assay. **C.** Representative color overlay to demonstrate the staining specificity.

Repeatability and Reproducibility of the Integrated Assay

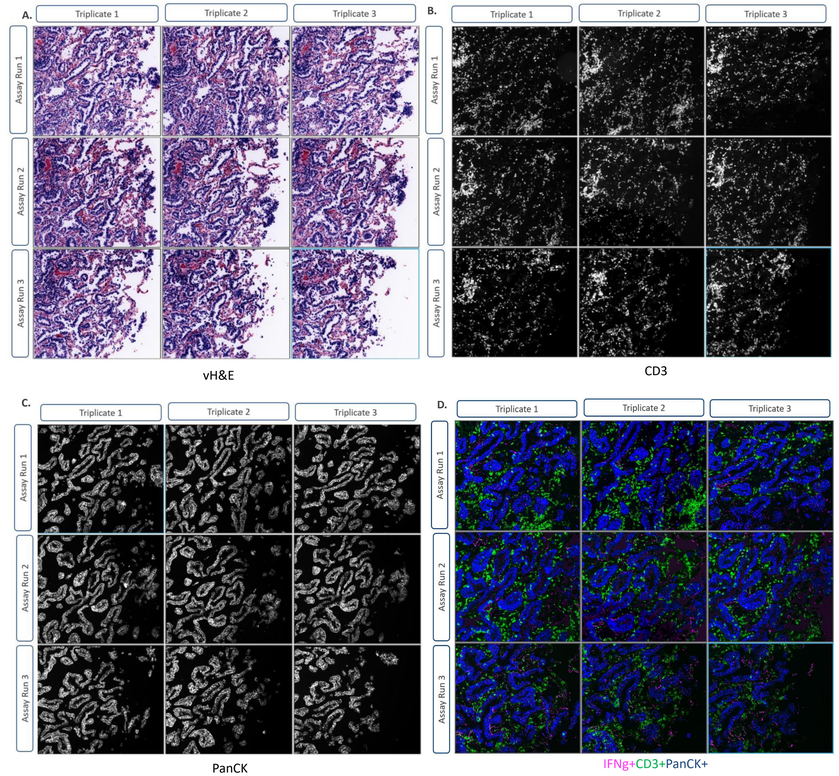
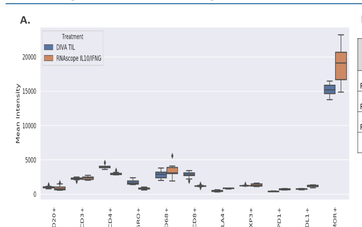


Figure 3. R&R of the biomarker staining in NSCLC sample in the integrated assay. A. vH&E (simulated H&E from fluorescence signals) of one the ROI in the R&R study; Representative biomarker staining in R&R runs are shown in B and C. D. Color overlaid images to show IFNg mRNA expression in tumor cells and T cells.

Analytics Results by NeoGenomics Imaging Analysis



B.

CV	CD3+ Mean Intensity	CD4+ Mean Intensity	CD45RO+ Mean Intensity	CD8+ Mean Intensity	CD68+ Mean Intensity	CTLA4+ Mean Intensity	CXCL10ISH + Mean Intensity	FOXP3+ Mean Intensity	IFNGISH+ Mean Intensity	PD1+ Mean Intensity	PDL1+ Mean Intensity	TUMOR+ Mean Intensity
Repeatability (Run 1)	7	4	2	6	3	4	0	8	1	6	5	6
Repeatability (Run 2)	7	10	11	14	24	2	1	5	1	15	9	8
Repeatability (Run 3)	3	3	10	11	21	1	3	5	2	6	9	19
Reproducibility	11	3	16	4	33	5	2	16	20	10	11	15

Figure 4. Analytics Results of Integrated Assay. A. Box plots to compare the biomarker intensity results between the integrated assay and IF assay. B. The intensity CV% of repeatability and reproducibility of each biomarker in the R&R run.

Key Findings

- The integrated RNAscope-MO IF workflow maintains accurate and robust staining accuracy for the TIL markers analyzed in this study and provides a novel, robust platform to combine RNA ISH with IF multiplexing.
- This integrated approach is shown to spatially correlate the distribution of cytokine RNA expression in immune cell subsets within the tumor microenvironment of NSCLC samples.
- Co-detection of RNA and protein provides critical information such as the source of secreted proteins (e.g. cytokines) or cell type specific transcript levels.
- The novel integrated RNAscope - MultiOmyx IF assay is a robust and sensitive platform for simultaneous detection of multiple RNA and protein biomarkers.

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