Simultaneous visualization of multiple immuno-oncology related markers in the tumor microenvironment by the RNAscope® LS Multiplex Fluorescent Assay

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Highlights

The RNAscope® assay is a highly sensitive and specific RNA ISH technology that identifies RNA expression at the single cell level with morphological context. Here we present the use of the RNAscope® Multiplex Fluorescent assay to visualize the interactions and spatial relationships between immune and tumor cells in the tumor microenvironment (TME).

Targets examined in this dataset include (Table 1):

- Checkpoint markers
- Tumor markers
- Immune cell markers
- Cytokines and chemokines

Detection of these RNA targets with the RNAscope® assay can aid in:

- Characterizing immune cell types and states
- Profiling immune checkpoint markers and other therapeutic targets in TME
- Understanding complex spatial relationships among different cell types within TME

Immuno-oncology is an innovative area of research and therapeutic development that aims at harnessing the patient's own immune system to help fight cancer[1,2]. Recent clinical successes with immune checkpoint blockade have provided promising immune-based therapeutic approaches for controlling malignancy. While therapeutic antibodies against CTLA-4 and PD-1/PD-L1 have resulted in potent and durable clinical responses in many patients, there still remains an urgent need to develop biomarker assays to identify patients who may benefit from these approaches. While several biomarker analysis technologies are available, most do not provide spatial and cell type-specific information critical for assessing the specific immune cell types with lineage and functional information in the evolving microenvironment of each tumor. Furthermore, multiplexing capabilities are highly desirable in order to obtain comprehensive single cell-level co-expression information and to maximize the use of limited biopsied sample material.

The RNAscope® technology is an advanced *in situ* hybridization assay that allows for the visualization of single-cell gene expression, targeting mRNA sequences directly in tissues[3]. The proprietary double Z probe design, in combination with the advanced signal amplification enables a highly specific and sensitive detection of target mRNAs and long non-coding RNAs in fresh frozen,

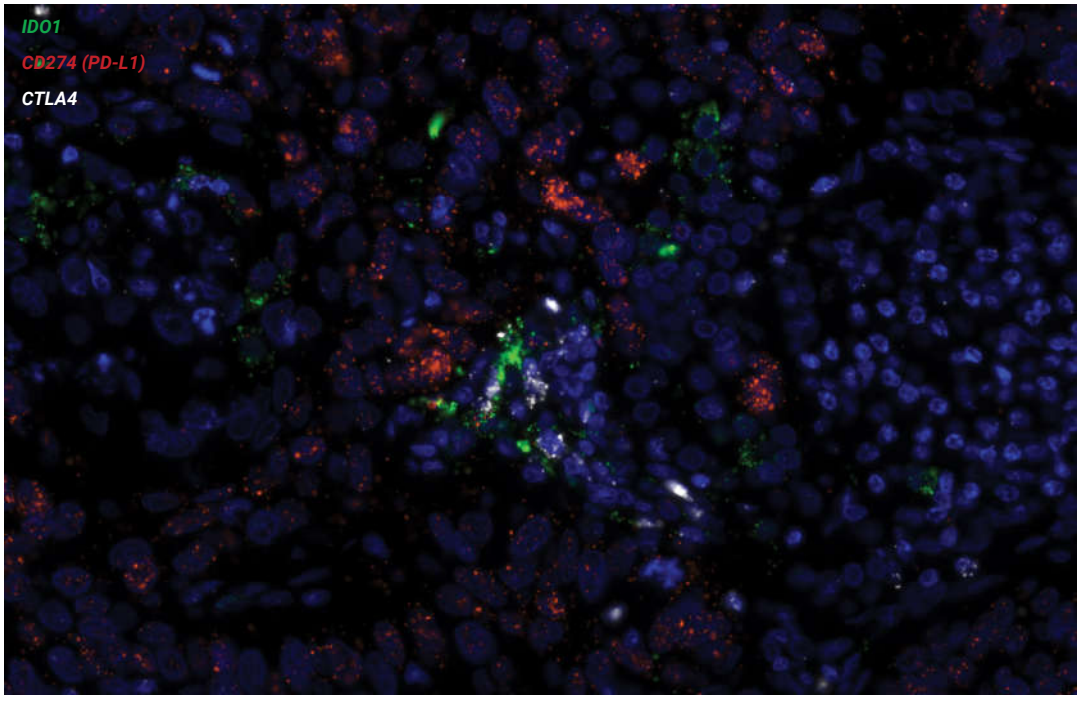

FIGURE 1. The RNAscope® LS Multiplex Fluorescent Assay for immuno-oncology studies. The RNAscope® LS Multiplex Fluorescent assay was used to simultaneously detect three markers in non-small cell lung cancer (NSCLC) tissue. Nuclei were stained using DAPI (blue).

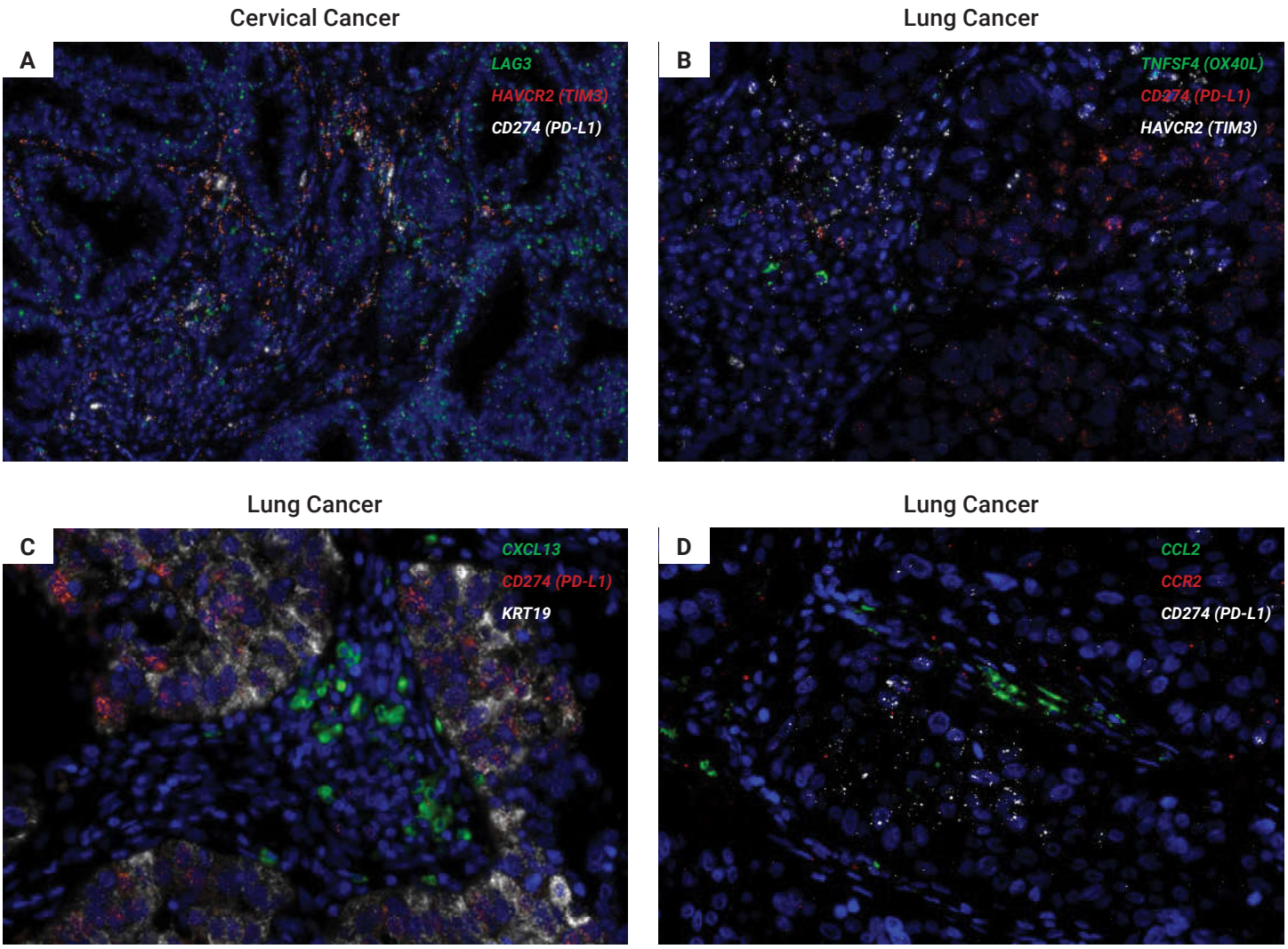

FIGURE 2. Simultaneous visualization of multiple immuno-oncology related markers in human cancer tissues. The RNAscope® LS Multiplex Fluorescent assay was used to simultaneously detect sets of three markers in cervical (A) and NSCLC (B–D) tissues. Nuclei were stained using DAPI (blue).

fixed frozen, and formalin-fixed paraffin-embedded (FFPE) cells and tissues, with each dot representing a single RNA transcript. Therefore, this robust signal-to-noise technology allows for the detection of gene transcripts at single molecule level with single-cell resolution. The multiplexing capabilities of the RNAscope® Multiplex Fluorescent assay, with simultaneous detection of up to 4 targets, provide pivotal single cell imaging data to gain better insights into the tumor immunology, and the complex and heterogeneous tumor microenvironment and its dynamics in cancer initiation, progression and metastasis.

In this report we utilized the RNAscope® LS Multiplex Fluorescent assay, performed on the Leica Biosystems' BOND RX automated staining system, to visualize the expression of multiple immune, tumor and checkpoint markers within the tumor microenvironment in order to:

- Characterize immune cell types and states
- Profile immune checkpoint markers and other therapeutic targets in TME
- Understand complex spatial relationship among different cell types within TME

Figures 1–3 demonstrate the use of the RNAscope® LS Multiplex Fluorescent assay in visualizing the interactions and spatial relationships between multiple cell types (eg. immune and tumor cell type markers), cytokines, and checkpoint markers, amongst others. Also, the possibility to combine ISH with subsequent immunohistochemistry (IHC) is exemplified in Figures 4 and 5, with Figure 4 showing the co-localization of *CD8a* mRNA and CD8 protein. In a recent publication by Sanmamed *et al.* 2017, the authors combined fluorescent RNAscope® ISH for *IL-8* with IHC for cytokeratin to quantitatively analyze *IL-8* mRNA expression in the tumor (cytokeratin-positive) and stromal (cytokeratin-negative) compartments of NSCLC samples[4].

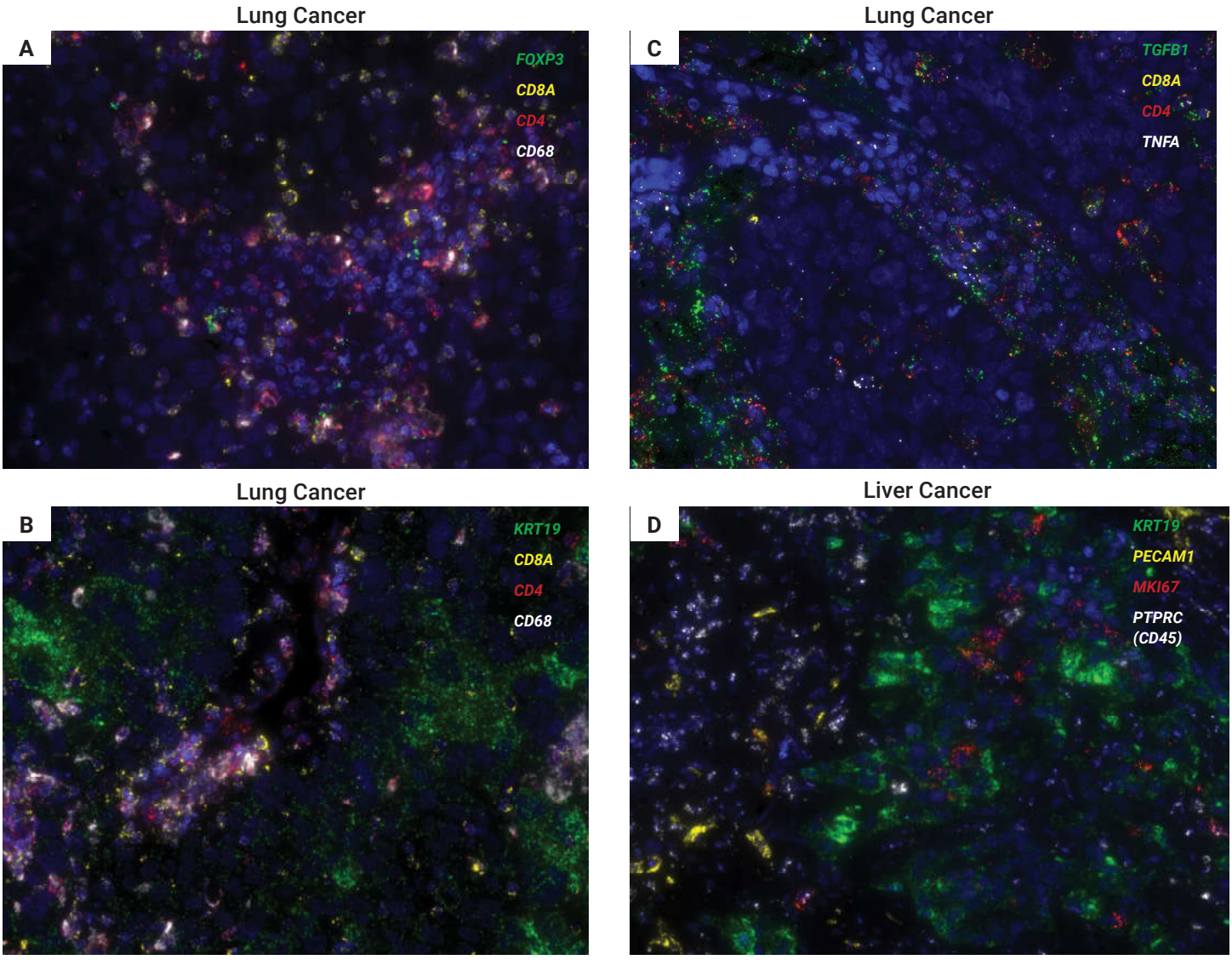

FIGURE 3. Simultaneous detection of 4 RNA targets in human cancer tissues. The RNAscope® LS Multiplex Fluorescent assay was used to simultaneously detect four markers in human NSCLC (A-C) and liver (D) tissues. Nuclei were stained using DAPI (blue).

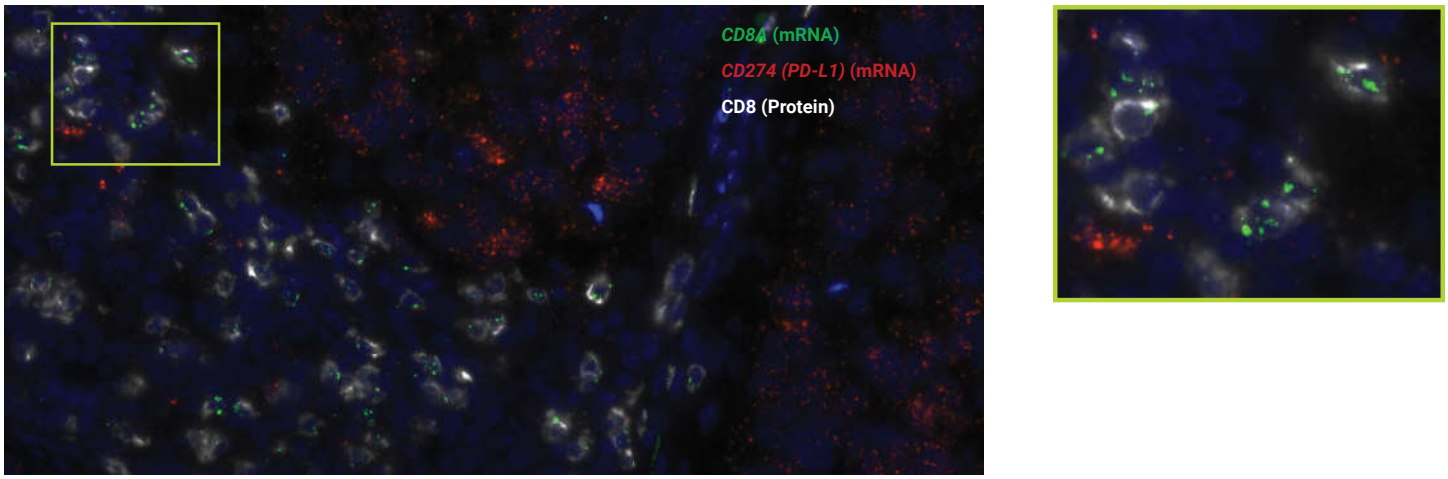

FIGURE 4. Dual ISH-IHC for the simultaneous detection of RNA and protein in human lung cancer. The RNAscope® LS Multiplex Fluorescent ISH assay can be combined with immunohistochemistry (IHC). The RNAscope® Multiplex Fluorescent assay was used to simultaneously detect *CD8A* (green) and *CD274* (*PD-L1*) (red) mRNA followed by IHC for the CD8 protein (white) in NSCLC tissue. (Inset) Co-localization was observed for the *CD8A* mRNA by ISH and the CD8 protein by IHC.

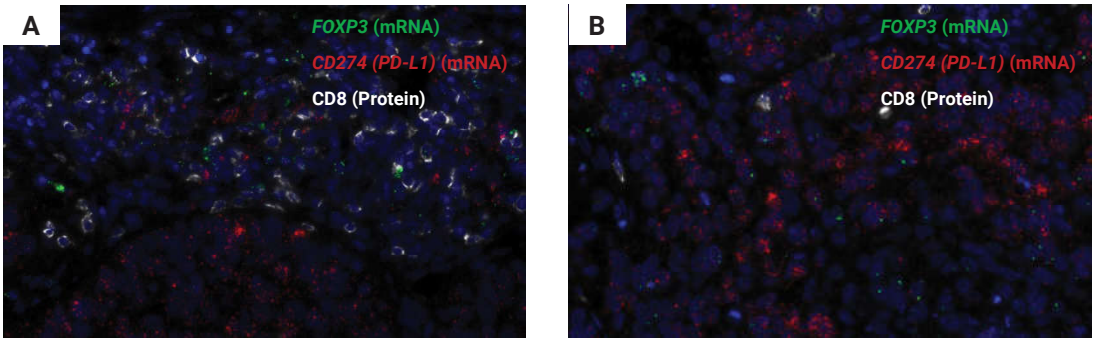

FIGURE 5. Dual ISH-IHC for the simultaneous detection of RNA and protein in human lung cancer. The RNAscope® LS Multiplex Fluorescent ISH assay can be combined with immunohistochemistry (IHC). The RNAscope® LS Multiplex Fluorescent assay was used to simultaneously detect two mRNA targets followed by IHC for the CD8 protein (white) in NSCLC tissue.

Conclusions

Detecting the expression of multiple RNA biomarkers at the single-cell level while preserving spatial information is critical to understanding tumor immunology and potential immunotherapy approaches. Here we show that the RNAscope® LS Multiplex Fluorescent assay is an ideal solution to detect multiple genes simultaneously (including chemokines, cytokines, and various checkpoint, tumor, and immune markers). Co-expression profiles of multiple checkpoint markers in the tumor microenvironment can reveal unique and heterogeneous patterns of expression in different tumor tissues. This information may reveal potential insights into combination therapies targeted against different checkpoint pathways. Furthermore, combining the RNAscope® LS Multiplex Fluorescent ISH assay with IHC provides the capability to visualize multiple RNA markers and immune cell markers in the tumor microenvironment.

References

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Markers	Probe Name	Cat No.
Checkpoint Markers		
IDO1	Hs-IDO1	602681
CTLA4	Hs-CTLA4	554341
CD274 (PD-L1)	Hs-CD274	600861
LAG3	Hs-LAG3	553931
HAVCR2 (TIM3)	Hs-HAVCR2	560681
Cytokines and Chemokines		
TGFβ1	Hs-TGFB1	400881
TNFα	Hs-TNFA	310421
CCL2	Hs-CCL2	423811
CCR2	Hs-CCR2	438221
CXCL13	Hs-CXCL13	311321
Immune Cell Markers		
CD4	Hs-CD4	605601
CD8A	Hs-CD8A	560391
CD68	Hs-CD68	560591
FOXP3	Hs-FOXP3	418471
PTPRC (CD45)	Hs-PTPRC	601991
TNFSF4 (OX40L)	Hs-TNFSF4	427201
Additional Markers		
KRT19	Hs-KRT19	310221
PECAM1	Hs-PECAM1	548451
MKI67	Hs-MKI67	591771

TABLE 1. Checkpoint markers, immune cell markers, chemokines, cytokines, and other markers examined in this dataset by the RNAscope® LS Multiplex Fluorescent assay.

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