Combining the best of both worlds: Immune Profiling the Tumor Microenvironment with RNA and Protein

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Background

Recent success in cancer immunotherapy have benefited many cancer patients across multiple malignancies, generating much interest from all sides. At the same time, there is an urgent need to develop predictive biomarkers to identify patients who are most likely to benefit from various immunotherapeutic strategies. While many 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.

In this study, we demonstrate the development of an improved fluorescence multiplex *in situ* hybridization (ISH) method to detect three RNA biomarkers simultaneously in FFPE and fresh frozen tissues on the Leica BOND RX automated slide staining system. The presented method detects RNA biomarkers in a highly specific and sensitive manner, overcoming the inherent challenge of auto-fluorescence in FFPE tissues.

Design

In this investigation of 60 archived formalin-fixed paraffin embedded (FFPE) non-small cell lung cancer (NSCLC) biopsies, expression profiles of checkpoint markers and immune functional molecules were evaluated in the tissue environment by RNAscope® ISH assay (Figure 1).

Tissue microarray (TMA) preparation and RNAscope analysis: FFPE tissues of 60 Lung carcinoma samples were assembled as TMAs as shown in Figure 3. Serial sections of slides were subjected to gene expression analysis by applying manual RNAscope 2.5 HD assay or newly developed fluorescence multiplex assay in combination with immunohistochemistry (Figure 2).

Combination of RNAscope and IHC for multiplex fluorescence analysis: Improved fluorescence multiplex RNAscope method to detect three RNA biomarkers or two RNA biomarkers plus one IHC target simultaneously in FFPE and fresh frozen tissues was developed on the Leica BOND RX automated slide staining system. Schematic workflow is shown in Figure 2.

Imaging and quantitation: Images were acquired using a Leica Biosystems Aperio AT2 Digital Pathology Slide Scanner or Zeiss microscope equipped with the Nuance Multispectral Imaging system. Chromogenic RNAscope signals were quantified by Indica Labs' HALO™ imaging analysis algorithm.

Figure 1. RNAscope® technology and workflow

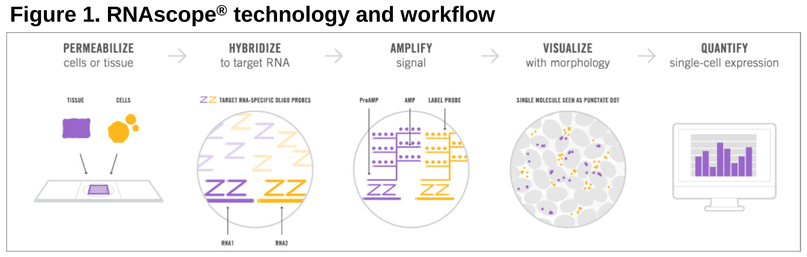
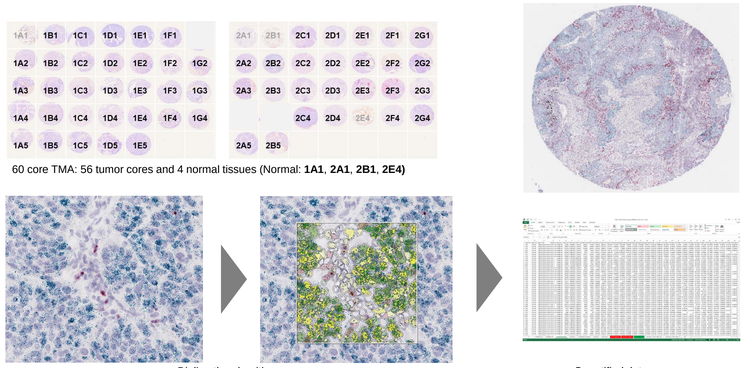


Figure 2. Schematic workflow of improved fluorescence multiplex assay in Leica BOND RX for simultaneous RNA and protein detection

Figure 3. Human NSCLC TMA schematic and HALO analysis



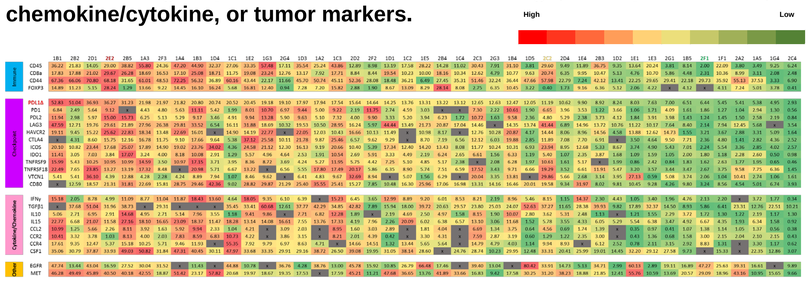
60 core TMA: 56 tumor cores and 4 normal tissues (Normal: **1A1**, **2A1**, **2B1**, **2E4**)

Dialing the algorithm

Quantified data

Results

Figure 4. Heat map of selected TMA cores representing percent positive cells expressing respective markers: immune lineage, checkpoint-related, chemokine/cytokine, or tumor markers.

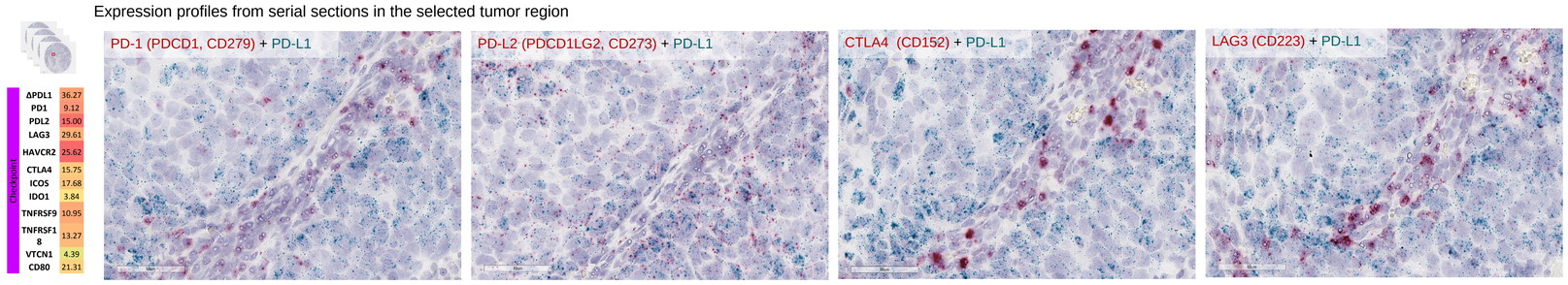


Data sorted based on %PDL1 positive cells. PDL1 values represent average from 18 separate assays.
Cores shaded in grey indicate tissues that were folded over or missing more than 50%.

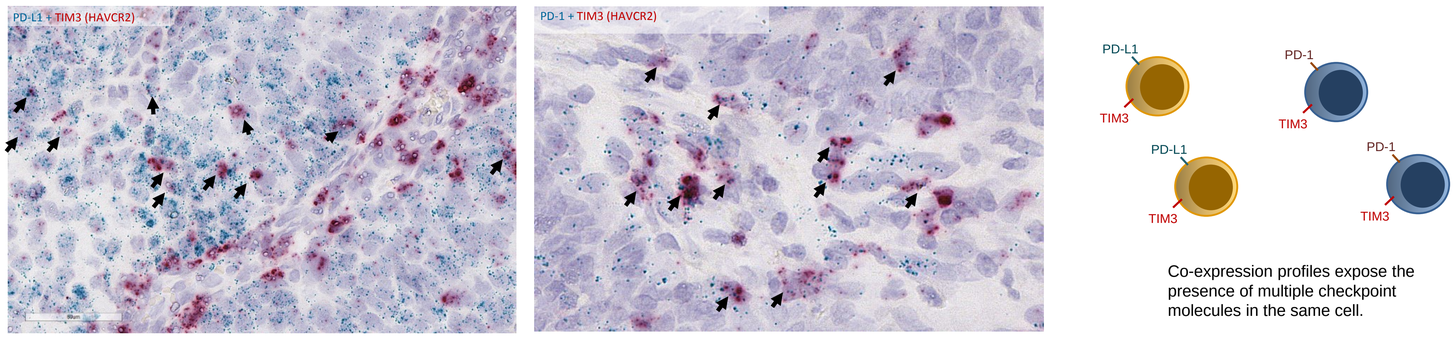
Results

Figure 5. Co-expression profiles of checkpoint markers in a tumor microenvironment (core 2E2)

Expression profiles from serial sections in the selected tumor region



Single cell analysis by RNAscope® duplex assay reveals co-expression of TIM3 with PD-1 or PD-L1 in the same cells. NSCLC TMA core 2E2

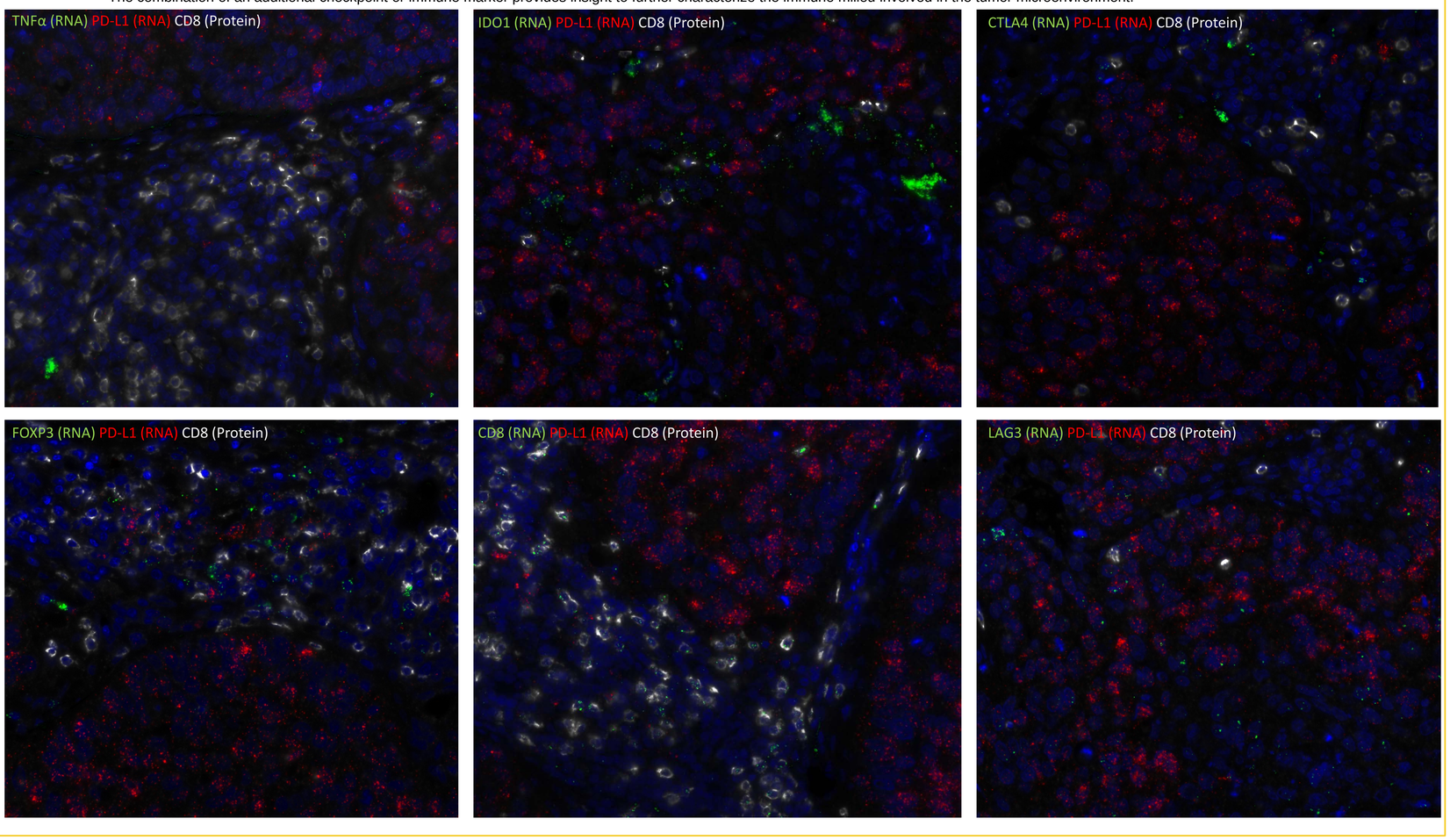


Co-expression profiles expose the presence of multiple checkpoint molecules in the same cell.

Cells co-expressing PD-1 and TIM3 or PD-L1 and TIM3 in the same cells

Figure 6. Immune infiltration visualized by fluorescence multiplex assay for simultaneous RNA and protein targets in FFPE NSCLC tissues (TMA core 1B1)

Infiltration of CD8+ cells, visualized via IHC, into tumor regions marked by high PD-L1 RNA is consistent with the heat map analysis.
The combination of an additional checkpoint or immune marker provides insight to further characterize the immune milieu involved in the tumor microenvironment.



Conclusions

The RNAscope® assay is a highly specific and sensitive RNA *in situ* hybridization technology that identifies gene expression at the single cell level while maintaining an intact environment to provide morphological context. In this study, the expression profiles of immune checkpoint and functional markers were evaluated in the tumor microenvironment of 60 NSCLC FFPE tissues by RNAscope® assay.

- **The immune infiltration in the tumor microenvironment, revealed by RNAscope® ISH, is readily quantifiable in addition to visual characterization of specific target RNA detection within the morphological context.**
- **Co-expression profiles of multiple checkpoint markers in the tumor microenvironment revealed a unique and heterogeneous pattern of expression in different tumor tissues. This information may reveal potential insights into combination therapies targeted against different checkpoint pathways.**
- **The improved fluorescence multiplex assay by automated LS BOND Rx system provides dual ISH and IHC capability to visualize multiple RNA markers and a immune lineage marker in the tumor microenvironment.**