Detection of immune cell checkpoint and functional markers in the tumor microenvironment by the RNA \textit{in situ} hybridization RNAscope\textsuperscript{®} assay

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**Introduction**

The field of cancer immunotherapy has expanded rapidly in recent years with immune checkpoint inhibitors and other therapeutic approaches such as cancer vaccines and chimeric antigen receptor (CAR) therapy showing promising clinical results. Despite the dramatic and durable responses seen in many patients, our understanding of the immune response to cancer is still limited, and we cannot reliably predict who will or will not benefit from these new interventions. To better stratify patients for immunotherapy treatments, the series of events and biomarkers involved in the cancer-immunity cycle need to be better understood\textsuperscript{(1-3)}. In addition, spatially mapped expression data at the single-cell level is crucial to understanding the cellular organization and cell-to-cell interactions in the tumor and its complex microenvironment (TME).

RNAscope\textsuperscript{®} is a unique RNA ISH technology that provides single-cell gene expression resolution with spatial and morphological context. The RNAscope\textsuperscript{®} assay detects mRNA and long non-coding RNAs in fresh frozen, fresh fixed, and formalin-fixed paraffin-embedded (FFPE) cells and tissues by utilizing a unique double Z probe design and signal amplification strategy that allows for visualization of target RNA as a single dot, where each dot is an individual RNA molecule\textsuperscript{(4)}. The RNAscope\textsuperscript{®} strategy offers the key benefits of high sensitivity due to the signal amplification method, and high specificity because of the double Z probe design, resulting in a high signal-to-noise ratio in many tissues.

In this report we examined 50 selected immune checkpoint and functional markers (summarized in Figure 1) to demonstrate the utility of the RNAscope assay for tumor immunology applications. With the RNAscope\textsuperscript{®} 2.5 HD duplex assay we demonstrate localization of PD-L1 and several immune markers in ovarian and lung cancers (Figures 2-4). Detection of 12 immune checkpoint markers, 14 immune cell markers, and 24 immune function markers, such as cytokines and chemokines, in multiple human tumors is also demonstrated by the RNAscope\textsuperscript{®} brown assay (Figures 5-7; Appendix). The complete data set can be viewed in the appendix available online at www.acdbio.com/immunotherapy.
FIGURE 1. Immune markers examined in this study by the RNAscope® assay. Fifty immune system markers were examined in this study, including 12 immune checkpoint markers, 14 immune cell markers, and 24 cytokines and chemokines.

- **Checkpoint markers**
  - BTLA (CD272)
  - CTLA4
  - IDO1
  - TIM3 (HAVCR2)
  - LAG3
  - MICA
  - MICB
  - PD-1 (PDCD1)
  - PD-L1 (CD274)
  - PD-L2 (PDCD1LG2)
  - 4-1BB/CD137 (TNFRSF9)
  - B7-H4 (VTCN1)

- **Immune cell markers**
  - CD3
  - CD8α (CD8A)
  - CD27
  - CD40
  - CD40L (CD40LG)
  - CD70
  - FOXP3
  - Granulysin (GNLY)
  - ICAM1
  - ICOS
  - Perforin (PRFI)
  - OX40L (TNFSF4)
  - OX40 (TNFRSF4)
  - HVEM (TNFRSF14)

- **Cytokines and chemokines**
  - CCL5
  - CCL7
  - CCL1
  - CCL13
  - CCL10
  - CCL13
  - IFNβ
  - IFNγ
  - IL1B
  - IL4
  - IL6
  - IL7
  - IL12
  - IL23
  - IL21
  - IL33
  - TGFβ

FIGURE 2. Simultaneous detection of PD-L1 and CD8α mRNAs. The manual RNAscope® 2.5 HD Duplex assay was used to detect expression of the immune checkpoint marker PD-L1 (green chromogen) and the immune cell marker CD8α (red chromogen) in human ovarian (A) and lung (B) tumors. Inset shows enlarged region outlined in white. Asterisk denotes PD-L1+/CD8- cells; arrowhead denotes PD-L1+/CD8+ cells. Note that in the lung cancer sample (B) PD-L1 is profusely expressed in both tumor cells and immune cells, with abundant CD8+ immune cell infiltration. However, in the ovarian cancer sample (A) PD-L1 expression is primarily restricted to the immune cells. S, stroma; T, tumor. White line delineates tumor margin. 40x magnification.
FIGURE 3. Simultaneous detection of IFNγ and immune cell markers CD3, CD8α and CD4 mRNAs. The manual RNAscope® 2.5 HD Duplex assay was used to detect expression of the immune function marker IFNγ and the immune cell markers CD3 (A-B), CD8α (C-D), or CD4 (E-F) in human ovarian (A, C, E) and lung (B, D, F) tumors. CD3, CD8α, and CD4 were detected using green chromogen and IFNγ was detected using red chromogen. Inset shows enlarged region outlined in white. Asterisk denotes CD+/IFNγ- cells; arrowhead denotes CD+/IFNγ+ cells; arrow denotes CD-/IFNγ+ cells. S, stroma; T, tumor. White line delineates tumor margin. 40x magnification.

FIGURE 4. Simultaneous detection of FOXP3 and CD4 mRNAs. The manual RNAscope® 2.5 HD Duplex assay was used to detect expression of the immune cell markers CD4 (green chromogen) and FOXP3 (red chromogen) in human ovarian (A) and lung (B) tumors. Inset shows enlarged region outlined in white. Asterisk denotes CD4+/FOXP3- cells; arrowhead denotes CD4+/FOXP3+ regulatory T cells. S, stroma; T, tumor. White line delineates tumor margin. 40x magnification.
FIGURE 5. Detection of immune checkpoint markers in human tumors. The RNAscope® manual brown assay was used to detect expression of several immune checkpoint markers in multiple human tumors. 40x magnification.
FIGURE 6. Detection of immune cell markers in human tumors. The RNAscope® manual brown assay was used to detect expression of several immune cell markers in multiple human tumors. 40x magnification.
FIGURE 7. Detection of cytokines, chemokines, and their receptors in human tumors. The RNAscope® manual brown assay was used to detect expression of several cytokines, chemokines, and their receptors in multiple human tumors. 40x magnification.
Conclusions

Detecting RNA biomarker expression at the single-cell level while preserving spatial information is critical to understanding cellular organization and cell-to-cell interactions in the cancer-immunity cycle. Here we show that the RNAscope® assay is able to detect immune checkpoint markers and immune function markers in a variety of human tumors. Overall these results demonstrate the utility of the RNAscope® technology in studying tumor immunology and key targets of immunotherapy in the tumor and its complex microenvironment.

Appendix containing all data discussed in this study is available at acdbio.com/immunotherapy
### Probes for duplex assay

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* Data not included in this report.

### References


**DISCLAIMER**

All samples used in this study have been qualified for RNA integrity with PPIB and dapB control probes before target probe analysis. Always qualify your samples as describe in the getting started section www.acdbio.com/technical-support/getting-started. You may observe a difference in staining pattern, due to variation in tissue preparation methods or associated biology within the tissue samples.