Advanced Cell Diagnostics

Detection of immune cell checkpoint and functional markers in the tumor microenvironment by the RNA *in situ* hybridization RNAscope® assay

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The RNAscope® assay is a unique RNA ISH technology that identifies RNA expression at the single cell level with morphological context. Here we present the use of RNAscope® for the detection of RNA targets in the tumor microenvironment (TME) that are involved in tumor immunology and immunotherapy:

- Checkpoint markers
- Immune cell markers
- Cytokines and chemokines

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

- Localization of specific immune cell types (i.e., cytotoxic lymphocytes and regulatory T cells) in the TME
- Determining spatial relationships between different cell types in the TME
- Characterization of secreted proteins (i.e., cytokines and chemokines)
- Evaluation of immune function in TME beyond enumeration of tumor infiltrating lymphocytes

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[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® is a unique RNA ISH technology that provides single-cell gene expression resolution with spatial and morphological context. The RNAscope® 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[4]. The RNAscope® 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® 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® brown assay (Figures 5-7; Appendix). The complete data set can be viewed in the appendix available online at www.acdbio.com/immunotherapy.

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 (PRF1)
OX40L (TNFSF4)
OX40 (TNFRSF4)
HVEM (TNFRSF14)

Cytokines and chemokines

CCL5
CCL7
CX3CL1
CX3CR1
CXCL9
CXCL10
CXCL13
IFNB1
IFNγ (IFNG)
IL1B
IL4
IL6
IL7
IL10
IL12A
IL13
IL17A
IL18
IL20
IL21
IL33
TGFβ (TGFB1)
TNFα (TNFA)
VEGF-A (VEGFA)

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.

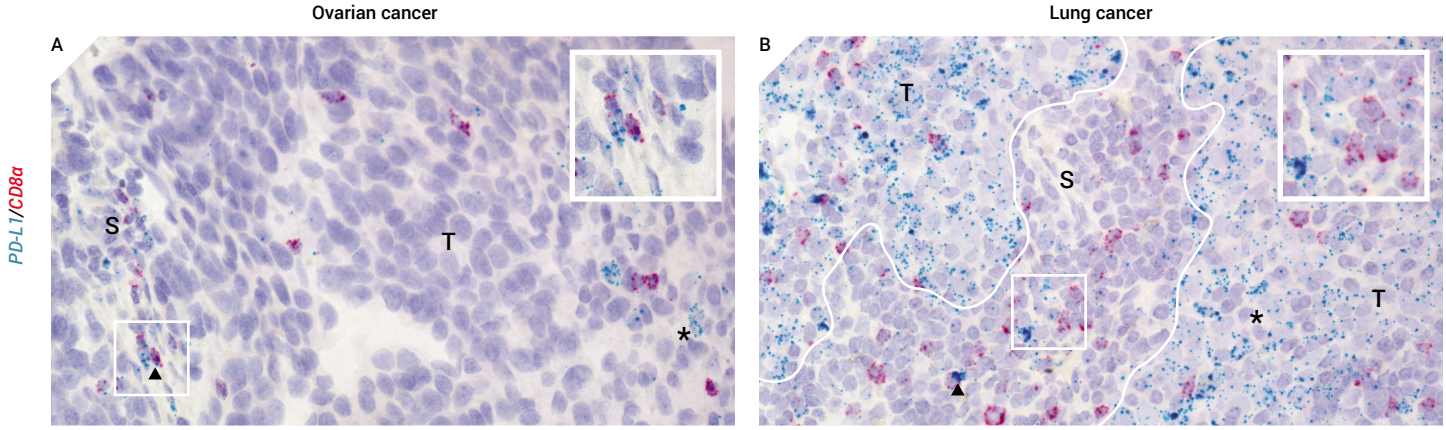

FIGURE 2. Simultaneous detection of *PD-L1* and *CD8α* mRNAs. The manual RNAscope® 2.5 HD Duplex assay was used to detect expression of the immune checkpoint marker *PD-L1* (green chromogen) and the immune cell marker *CD8α* (red chromogen) in human ovarian (A) and lung (B) tumors. Inset shows enlarged 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.

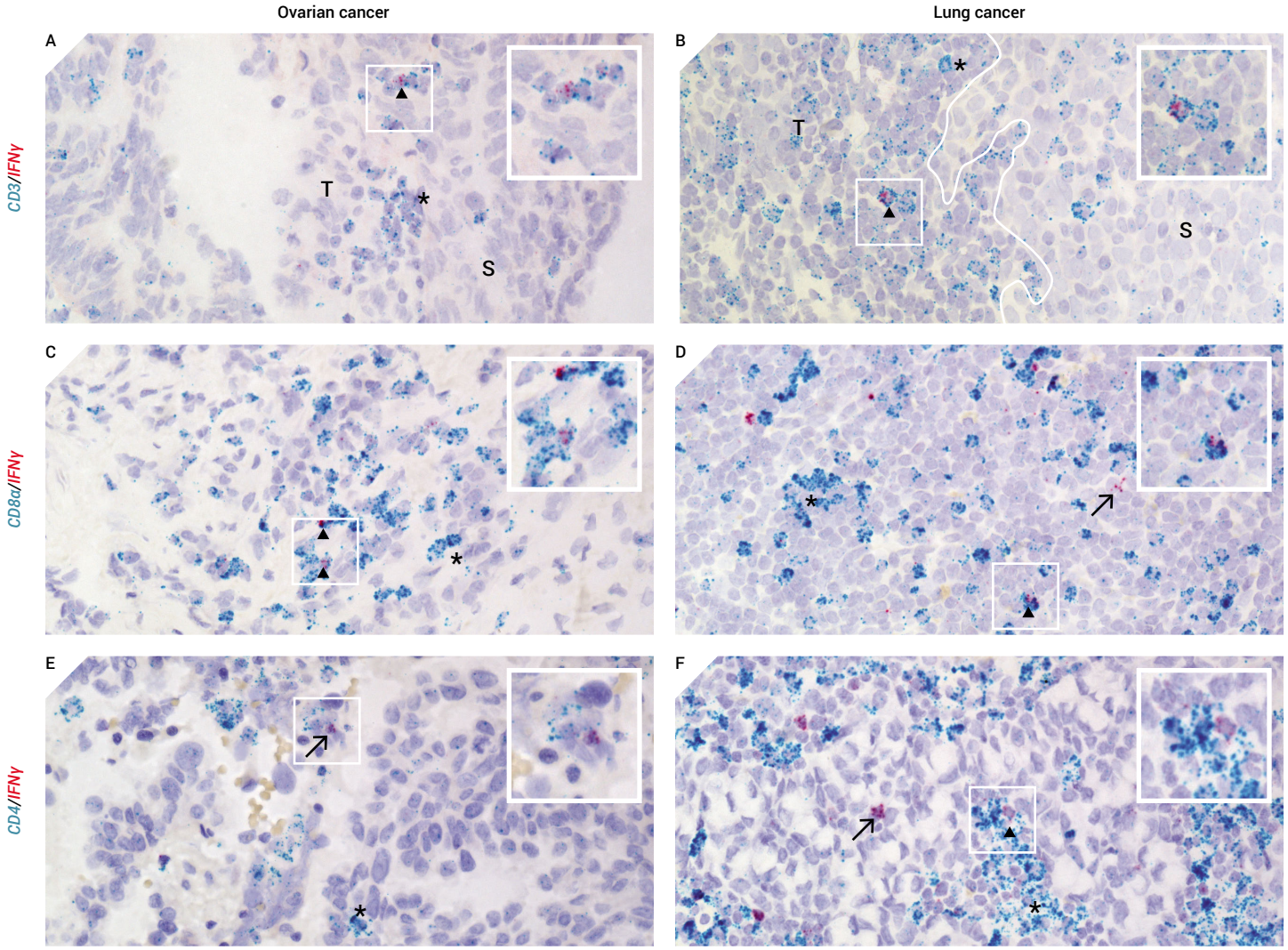

FIGURE 3. Simultaneous detection of *IFNγ* and immune cell markers *CD3, CD8α* and *CD4* mRNAs. The manual RNAscope® 2.5 HD Duplex assay was used to detect expression of the immune function marker *IFNγ* and the immune cell markers *CD3* (A-B), *CD8α* (C-D), or *CD4* (E-F) in human ovarian (A, C, E) 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.

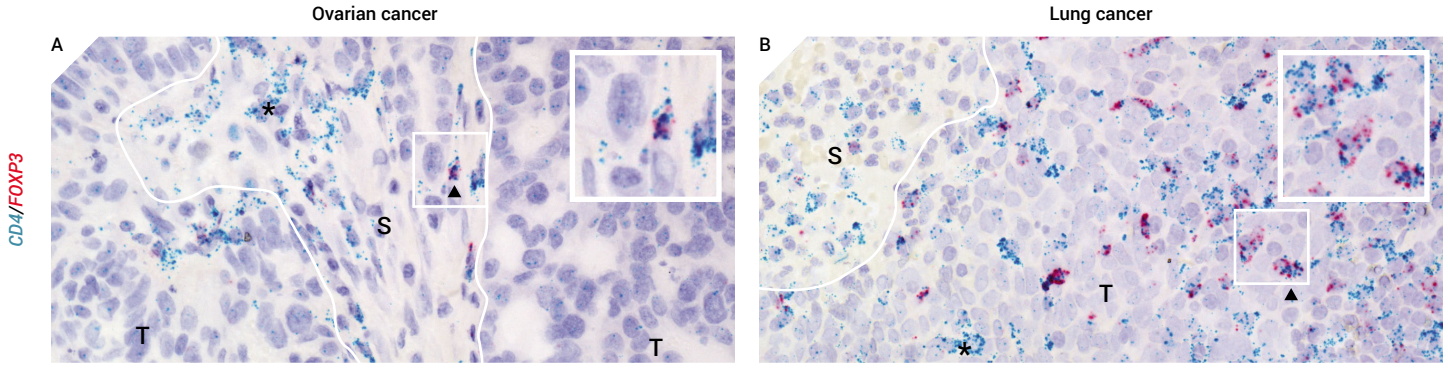

FIGURE 4. Simultaneous detection of *FOXP3* and *CD4* mRNAs. The manual RNAscope® 2.5 HD Duplex assay was used to detect expression of the immune cell markers *CD4* (green chromogen) and *FOXP3* (red chromogen) in human ovarian (A) and lung (B) tumors. Inset shows enlarged region outlined in white. Asterisk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

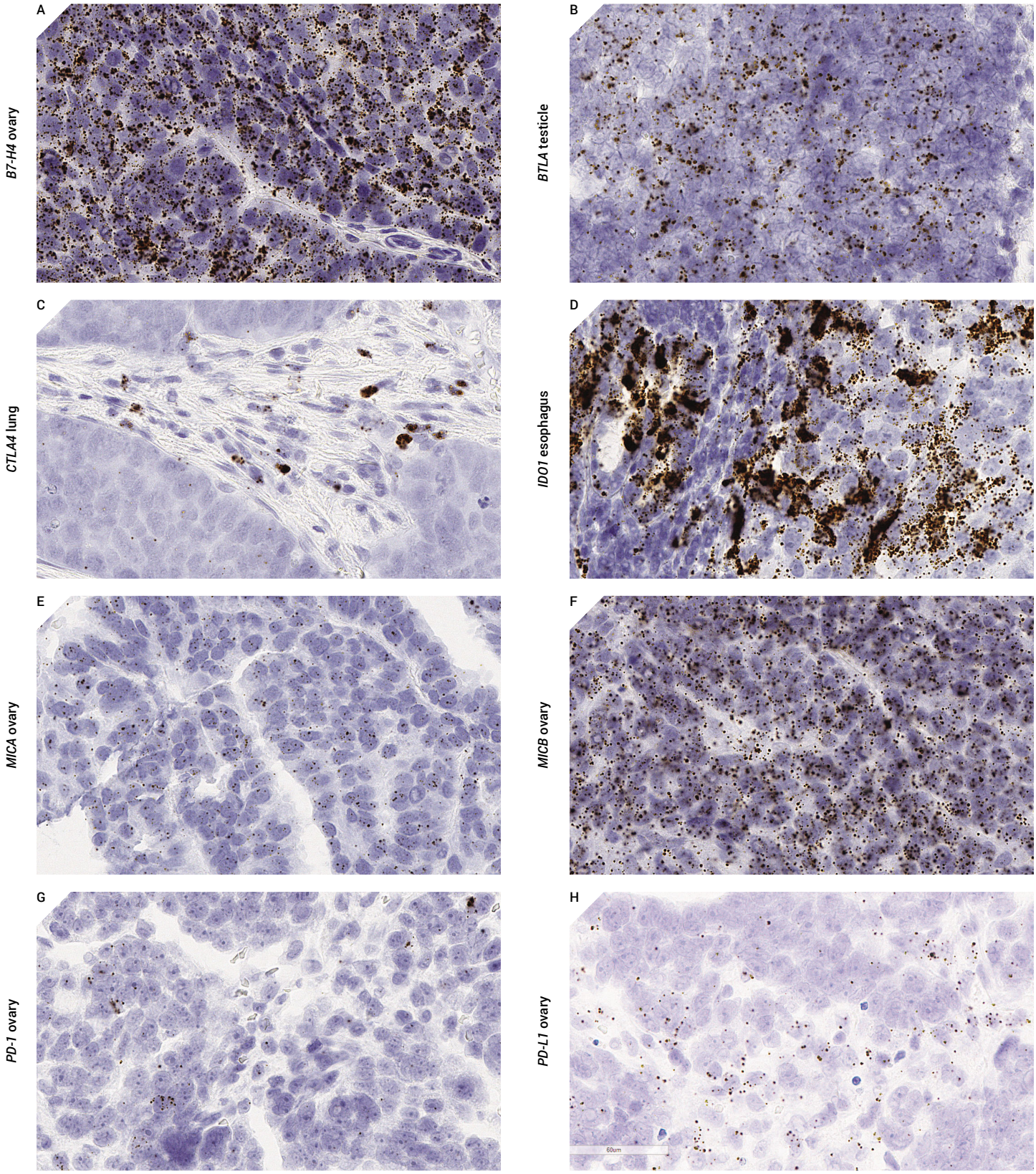

FIGURE 5. Detection of immune checkpoint markers in human tumors. The RNAscope® manual brown assay was used to detect expression of several immune checkpoint markers in multiple human tumors. 40x magnification.

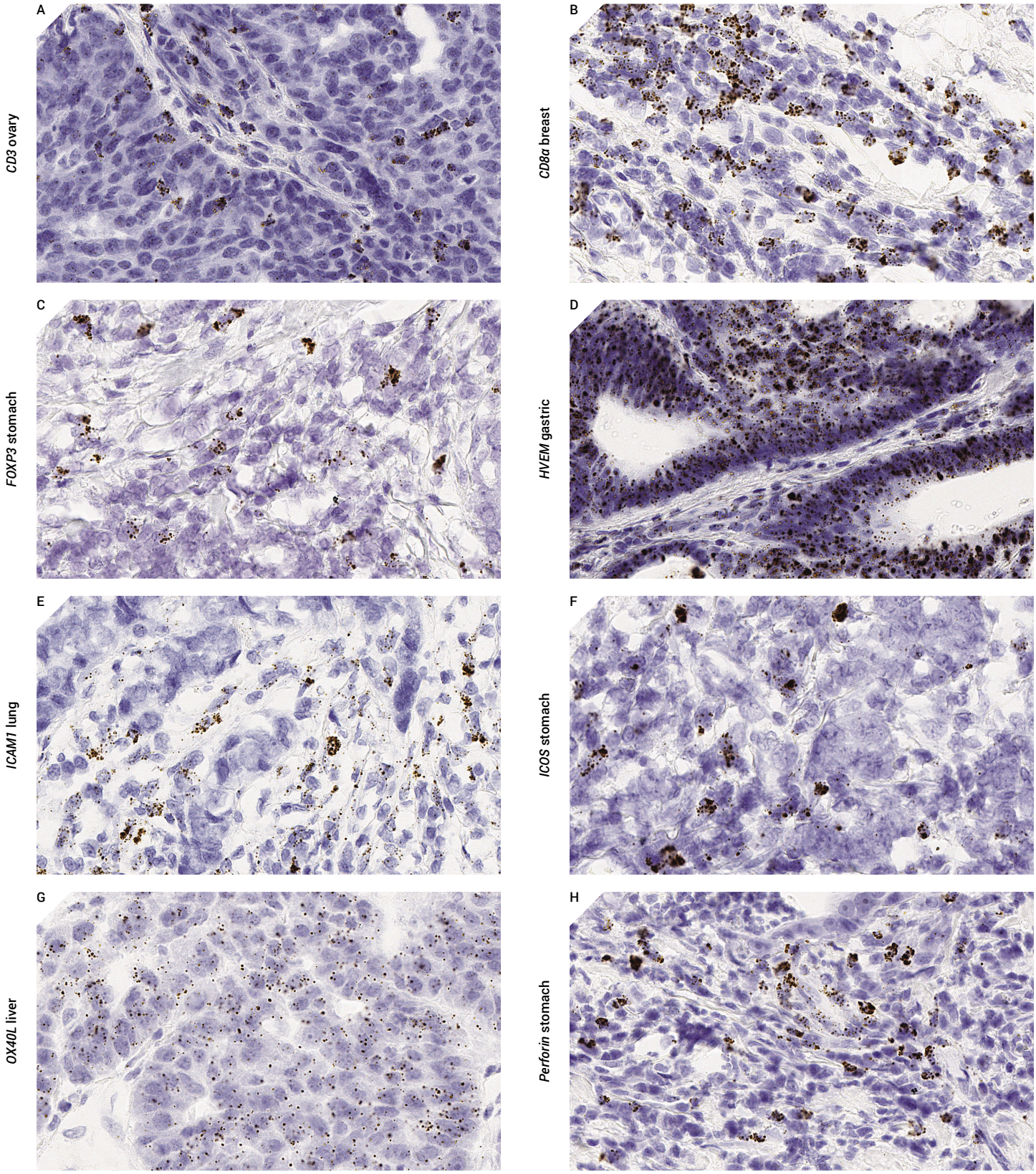

FIGURE 6. Detection of immune cell markers in human tumors. The RNAscope® manual brown assay was used to detect expression of several immune cell markers in multiple human tumors. 40x magnification.

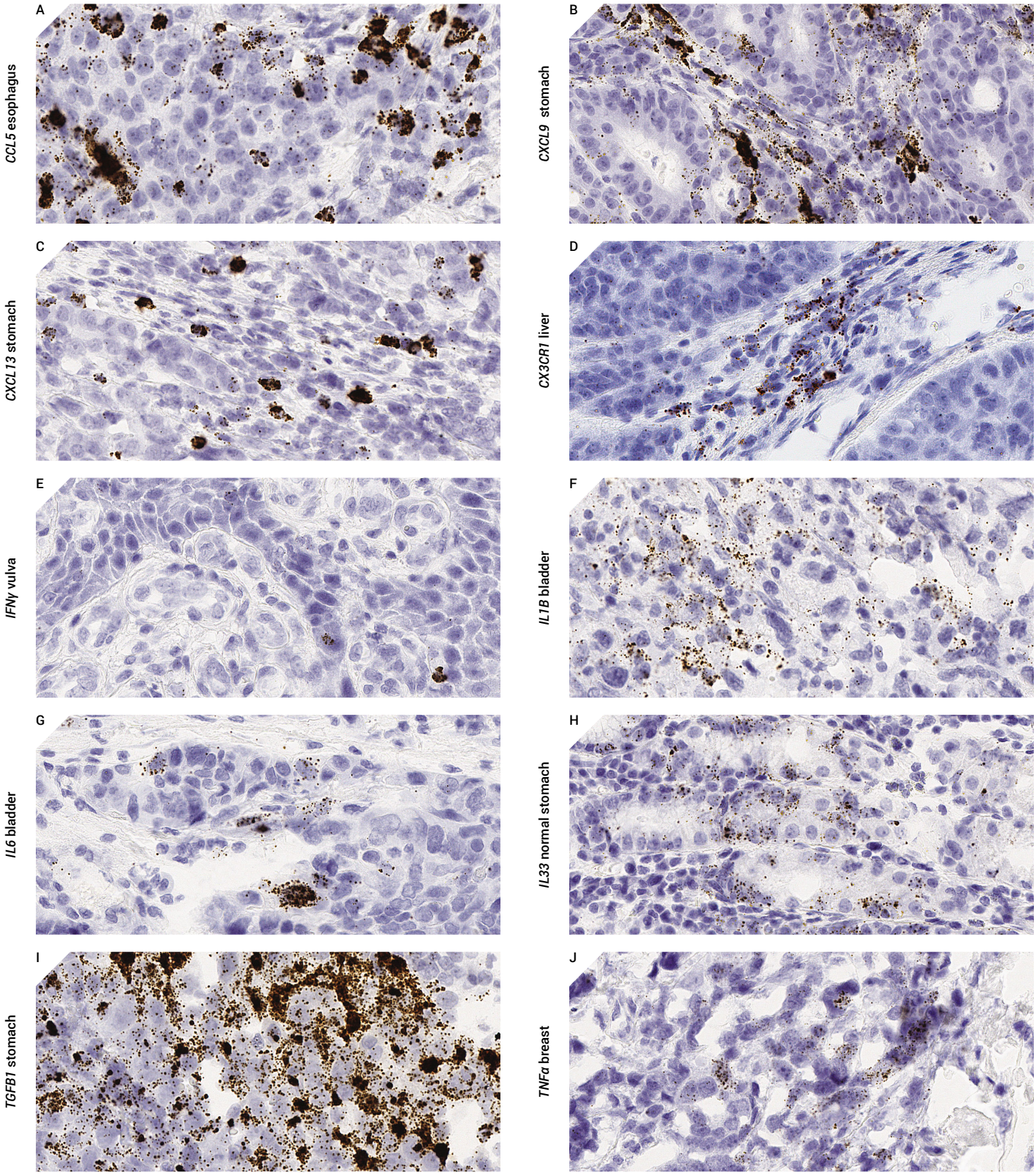

FIGURE 7. Detection of cytokines, chemokines, and their receptors in human tumors. The RNAscope® manual brown assay was used to detect expression of several cytokines, chemokines, and their receptors in multiple human tumors. 40x magnification.

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

Markers	Probe name	Cat No.
Checkpoint markers		
B7-H4 (VTCN1)	Hs-VTCN1	418081
BTLA (CD272)	Hs-BTLA	401601
CD137/4-1BB (TNFRSF9)	Hs-TNFRSF9	415171
CTLA4	Hs-CTLA4	554341
IDO1	Hs-IDO1	602681
TIM3 (HAVCR2)	Hs-HAVCR2	560681
LAG3	Hs-LAG3	553931
MICA	Hs-MICA	427161
MICB	Hs-MICB	427181
PD-1 (PDCD1)	Hs-PDCD1	602021
PD-L1 (CD274)	Hs-CD274	600861
PD-L2 (PDCD1LG2)	Hs-PDCD1LG2	551891
Immune cell markers		
CD3	Hs-CD3-pool	426621
CD8α (CD8A)	Hs-CD8A	560391
CD27	Hs-CD27	415451
CD40	Hs-CD40	578471
CD40L (CD40LG)	Hs-CD40LG	542341
CD70	Hs-CD70	419331
FOXP3	Hs-FOXP3	418471
Granulysin (GNLY)	Hs-GNLY	407371
HVEM (TNFRSF14)	Hs-TNFRSF14	319731
ICAM1	Hs-ICAM1	402951
ICOS	Hs-ICOS	407141
OX40L (TNFSF4)	Hs-TNFSF4	427201
OX40 (TNFRSF4)	Hs-TNFRSF4	412381
Perforin (PRF1)	Hs-PRF1	407381

Markers	Probe name	Cat No.
Cytokines and chemokines		
CCL5	Hs-CCL5	549171
CCL7	Hs-CCL7	425261
CX3CL1	Hs-CX3CL1	411261
CX3CR1	Hs-CX3CR1	411251
CXCL9	Hs-CXCL9	440161
CXCL10	Hs-CXCL10	311851
CXCL13	Hs-CXCL13	311321
IFNB1	Hs-IFNB1	417071
IFN-γ (IFNG)	Hs-IFNG	310501
IL1B	Hs-IL1B	310361
IL4	Hs-IL4	315191
IL6	Hs-IL6	310371
IL7	Hs-IL7	424251
IL10	Hs-IL10	602051
IL12A	Hs-IL12A	402061
IL13	Hs-IL13	586241
IL17A	Hs-IL17A	310931
IL18	Hs-IL18	400301
IL20	Hs-IL20	412201
IL21	Hs-IL21	401251
IL33	Hs-IL33	400111
TGF-β (TGFB1)	Hs-TGFB1	400881
TNF-α (TNFA)	Hs-TNFA	310421
VEGF-A (VEGFA)	Hs-VEGFA	423161

Probes for duplex assay		
Markers	**Probe name**	**Cat #**
PD-L1/CD8A	Hs-CD274	600681
	Hs-CD8A-C2	560391-C2
*PD-L1/CD3**	Hs-CD274	600681
	Hs-CD3-pool-C2	426621-C2
CD3/IFNG	Hs-CD3-pool	426621
	Hs-IFNG-C2	310501-C2
CD8a/IFNG	Hs-CD8A	560391
	Hs-IFNG-C2	310501-C2
CD4/IFNG	Hs-CD4	605601
	Hs-IFNG-C2	310501-C2
CD4/FOXP3	Hs-CD4	605601
	Hs-FOXP3-C2	418471-C2

* Data not included in this report.

References

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2. Chen DS and Mellman I. Oncology meets immunology: the cancer-immunity cycle. Immunity. 2013; 39(1): 1-10.
3. Tumeh PC et al. PD-1 blockade induces new responses by inhibiting adaptive immune response. Nature. 2014; 515(7528): 568-571.
4. Wang F, Flanagan J, Su N, Wang LC, Bui S, Nielson A, Wu X, Vo HT, Ma XJ, Luo Y. RNAscope: A novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. J Mol Diagn. 2012; 14(1):22-9.

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.

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