92 **SINGLE CELL AND SPATIAL MULTIPLEX PROFILING OF IMMUNE CELL MARKERS IN FFPE TUMOR TISSUES USING THE NOVEL RNASCOPE™ HIPLEX V2 IN SITU HYBRIDIZATION ASSAY**

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Background The tumor microenvironment (TME) is highly complex, comprised of tumor cells, immune cells, stromal cells, and extracellular matrix. Understanding spatial interactions between various cell types and their activation states in the TME is crucial for implementing successful immunotherapy strategies against various types of cancer. This study demonstrates a highly sensitive and specific multiplexed technique, the RNAscope HiPlex v2 in situ hybridization (ISH) assay for spatial and transcriptomic profiling of target genes to assess immune regulation in human lung, breast, cervical and ovarian FFPE tumor tissues.

Methods We have expanded our current RNAscope HiPlex assay capability of iteratively multiplexing up to 12 targets in fixed and fresh frozen samples to include formalin fixed paraffin embedded (FFPE) tissues. The novel FFPE reagent effectively reduces background autofluorescence, improving the signal to noise ratio. We have leveraged this technology to investigate spatial expression of 12 oncology and immuno-oncology target genes, including tumor markers, immune checkpoint markers, immunosuppression markers, immune cell markers and secreted chemokine RNA expression profile within the TME. The targets were simultaneously registered using HiPlex image registration software v2 that enables background subtraction.

Results We visualized T cell infiltration and identified T cell subsets within tumors using CD3and CD8 expression and activated T cells by IFNG expression. We further identified subsets of pro- and anti-inflammatory macrophages by CD68 and CD163 expression as well effector cells which secrete chemokines and cytokine. We also detected the hypoxia markers HIF1A and VEGF to elucidate the immunosuppressive state of tumor cells. Preliminary analysis and quantification of the HIF1A expression using HALO® image analysis software showed higher copy numbers in the lung tumor as compared to the other tumors, demonstrating the sensitivity of the assay through differential expression. We additionally showed the differential expression of immune checkpoint markers PDCD1, and CD274 within the TME.

Conclusions Using a highly sensitive multiplexed RNAscope HiPlex v2 ISH assay, we have demonstrated the capability of this technique to spatially resolve 12 targets in four different tumor types. The FFPE reagent efficiently quenched background autofluorescence in the tissues and identified immune cell signatures within the TME. Quantification of immunosuppressive markers further depicted a differential expression among various tumors. This technology is highly beneficial for investigating complex and spatial tumor-stroma interactions in basic science and translational research. The assay can also provide valuable understanding of the biological crosstalk among various cell types in complex and heterogeneous tissues.

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