Spatial multiplex profiling of immune cell markers in FFPE tumor tissues using the RNAscope™ HiPlex v2 in situ hybridization assay

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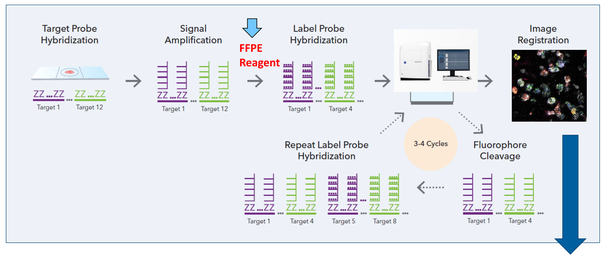
INTRODUCTION

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. Here we demonstrate a newly developed HiPlex v2 assay with capability of iteratively multiplexing up to 12-48 targets in FFPE, fixed and fresh frozen samples. Using a novel FFPE reagent that effectively reduces background autofluorescence and improves the signal to noise ratio, we investigated 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. Using this assay, we visualized T cell infiltration and identified T cell subsets within 4 different tumors using *CD3* and *CD8* expression and activated T cells by *IFNG* expression. Furthermore, we identified subsets of pro- and anti-inflammatory macrophages by *CD68* and *CD163* expression as well effector cells which secrete chemokines and cytokine. Additionally, we detected the hypoxia markers *HIF1A* and *VEGFA* to elucidate the immunosuppressive state of tumor cells. Further, we demonstrate how the HALO® image analysis platform can be employed with HiPlex v2 to quantitatively assess differential expression of 12 RNA targets, quantify distinct cell phenotypes based on gene expression, and to analyze spatial relationships between cell phenotypes within the TME. Quantification of T cell markers, Tumor Associated Macrophages (TAMs) and immunosuppressive markers using HALO® image analysis software depicted a differential expression among various tumors.

In conclusion, this study demonstrates a highly sensitive and specific multiplexed technique, the RNAscope HiPlex v2 ISH assay for gene expression profiling of target genes to assess immune regulation in FFPE tumor tissues. By expanding HiPlex capabilities to FFPE tissues, RNAscope HiPlex v2 image analysis and quantification provides a highly resolved spatial multiplexing solution to interrogate complex tissues and investigate biologically meaningful questions.

METHODS

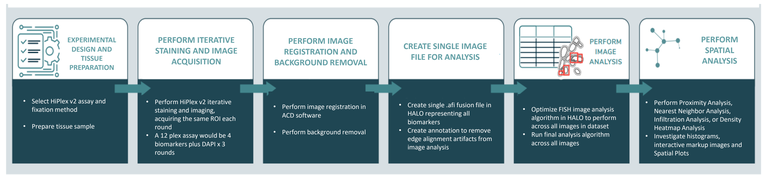
Iterative method of detecting 12 targets on the same tissue section



Assay: RNAscope HiPlex v2

- Samples: Tumor TMA
- Target Probes
- Positive control
- Negative control

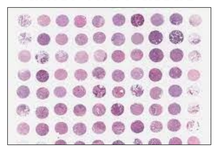
Quantitative HALO-HiPlex v2 Workflow



Probe Combinations (ACD HiPlex v2 probes T1-T12)

Cell types	Probes
Macrophages	***CD68 (T10), CD163 (T11), CCL22 (T1)***
T cells	***CD3 (T8), CD8 (T12) IFNG (T3),***
Tumor cells	***HIF1A (T7), PD-L1 (T9), CD3 (T8), VEGF (T4)***
Hypoxia marker	***HIF1A (T7)***

Tumor TMA tissue



RNAscope HiPlex v2 assay and HALO quantification workflow. The RNAscope HiPlex assay provides signal amplification for simultaneous visualization of up to 12 RNA targets in FFPE, fresh and fixed frozen samples by performing iterative fluorescent imaging detection. Images are registered using RNAscope image registration software followed by HALO image analysis.

RESULTS

1. FFPE reagent effectively quenches background autofluorescence and enhances visualization target RNA signals

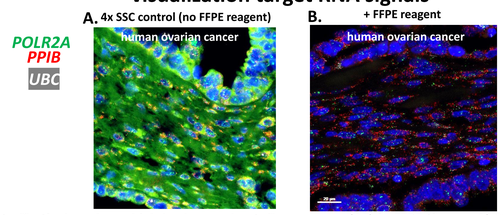


FIGURE 1. FFPE reagent effectively reduces high background autofluorescence (AF) in FFPE tissues. A) Standard HiPlex v1 assay without the FFPE reagent shows very high background autofluorescence. **B)** Introduction of the FFPE reagent successfully suppresses the AF in the FFPE tissues and enhances visualization of different target RNA signals indicating the effectiveness of the FFPE reagent to supress background AF.

2. The HiPlex v2 assay enables visualization of active immune cells, cytokines and chemokines within the TME

CD3 CD8 IFNG PD1 CD68 CD163 ARG1 CCL22 CXCL10 VEGFA HIF1A PDL1

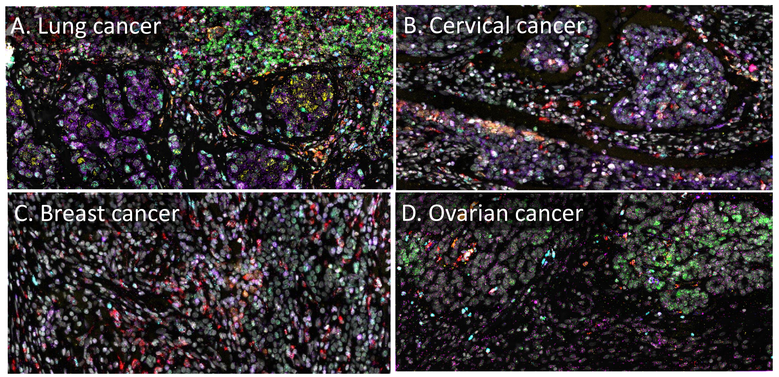


FIGURE 2. Profiling different cell types within the TME. 12 target specific marker probes were used to detect immune cells, tumor cells, chemokines and cytokines in **A)** Lung cancer, **B)** Cervical cancer, **C)** Breast cancer and **D)** Ovarian cancer. Nuclei stained with DAPI (gray).

3. Detecting infiltrated T cells subsets within tumors

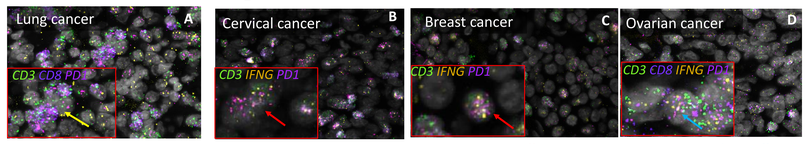


FIGURE 3. Detecting infiltrated T cells subsets within tumors. Target probes for *CD3*, *CD8*, *IFNG* and *PD1* were used to identify different subset of T cells in **A)** lung cancer, **B)** cervical cancer, **C)** breast cancer, **D)** ovarian cancer. *CD3+/CD8+/PD1+/IFNG+* cytotoxic T cells ↑ *CD3+/PD1+/IFNG+* T cells ↑ and *CD3+/CD8+/PD1+* T cells ↓. Nuclei stained with DAPI (gray).

4. Detecting macrophages markers, chemokines and cytokines within tumors

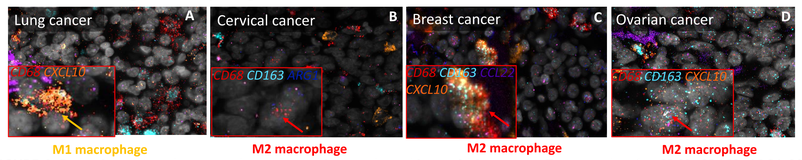


FIGURE 4. Detecting macrophages markers, chemokines and cytokines within tumors. Target probes for *CD68, CD163, ARG1 CCL22, CXCL10* were used to detect macrophages and visualize the cellular source of secreted factors in **A)** Lung cancer, **B)** cervical cancer, **C)** breast cancer, **D)** Ovarian cancer. *CD68+/ CD163+/ ARG1+/ CCL22+/ CXCL10+, CD68+/ CD163+/ CCL22+/ CXCL10+, CD68+/ CD163+/ ARG1+* M2 macrophages ↑ and CD68+/ CXCL10+ M1 ↑ macrophages were detected . Nuclei stained with DAPI (gray).

5. Image registration using RNAscope HiPlex v2 software and quantification using HALO® image analysis software

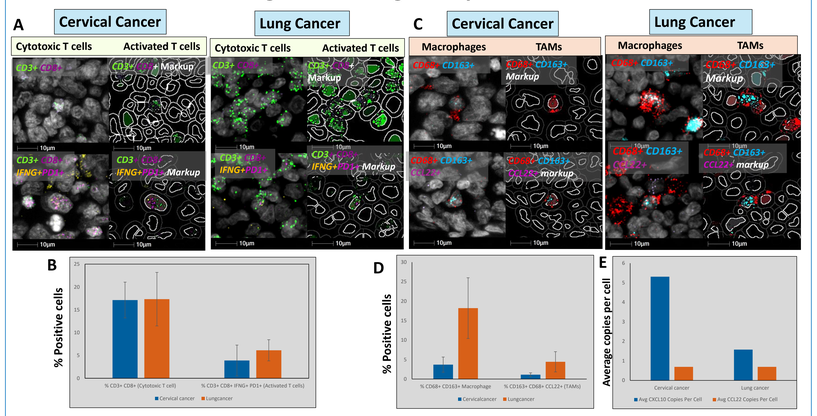


FIGURE 5. Detecting infiltrated T cells subsets and TAMs within tumors.
(A) Representative *CD3+ CD8+* and *CD3+ CD8+ IFNG+ PD1+* cells and the corresponding HALO markup is shown for each phenotype for cervical and lung cancer samples. The images were analyzed using the FISH module of the HALO software and all parameters were kept the same in the analysis of all ROIs (3 ROIs/tumor). The HALO markup image for each phenotype markup shows cells that do not meet the phenotypic requirements as an empty nucleus and empty cytoplasm. Cells that meet the phenotypic requirement are shown with the color markup. **(B)** Graph shows quantification of *CD3+CD8+* cytotoxic T cells, and *CD3+CD8+ IFNG+ PD1+* activated T cells using the HALO FISH module. **(C)** Target probes for *CD68, CD163, ARG1 CCL22, CXCL10* were used to detect macrophages and TAMs and visualize the cellular source of secreted factors in cervical cancer and lung cancer. Representative *CD68+ CD163+* macrophages and *CD68+ CD163+ CCL22+* TAM cells and the corresponding HALO markup is shown for each phenotype. **(D)** Quantification of *CD68+ CD163+* (total macrophages) and TAMs using HALO FISH module. **(E)** Average copy number per cell of secreted cytokines *CXCL10* and *CCL22* in cervical and lung cancer.

6. HALO Proximity Analysis- Tool to explore spatial relationships between immune cells and tumor cells

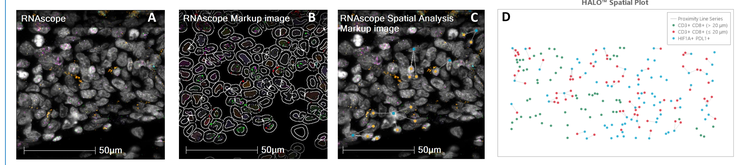


FIGURE 6. Proximity Analysis of *CD3+ CD8+* T cells within 20 µm of *HIF1A+ PD-L1+* tumor cells in cervical cancer tissue. A) 12-plex RNAscope image. **B)** FISH analysis on a 12-plex RNAscope image and Object Data is acquired. The 12-plex markup image is shown. **C)** Object Data from the FISH analysis is used in the Proximity Analysis and an interactive HALO markup image is shown where *HIF1A+ PD-L1+* are represented in blue and *CD3+ CD8+* cells that are less than or equal to 20 µm away are shown in yellow. **D)** In addition to an interactive markup image, HALO creates a Spatial Plot of the region analyzed. A Spatial Plot of an ROI is shown here where lines are drawn between *CD3+ CD8+* cells and *HIF1A+ PD-L1+* cells that are less than or equal to 20 µm away from one another. *CD3+ CD8+* cells that are less than or equal to 20 µm away from the *HIF1A+ PD-L1+* cells are shown in red and those that are greater than 20 µm away are shown in green. *HIF1A+ PD-L1+* cells are blue.

SUMMARY

- The new HiPlex v2 assay allows detection of 12 target RNA markers in FFPE tumor tissues without significant autofluorescence on the same section. Introduction of the FFPE reagent effectively suppresses autofluorescence background in FFPE tissues.
- The targets were simultaneously registered using RNAscope HiPlex image registration software v2 which effectively removes background from the registered images.
- Quantification and data analysis using HALO FISH module indicated differential expression of T cell and macrophage populations in the TME.
- Spatial Analysis was further performed on FISH Object Data in HALO. Proximity analysis examining the proximity of T cells and tumor cells in cervical cancer, indicated T-cell recruitment in tumors.

CONCLUSION

In conclusion, the HiPlex v2 assay is a powerful tool to reveal spatial organization of cells and provide deeper insight into the functions and phenotypes of novel cell populations. By expanding HiPlex capabilities to FFPE tissues, RNAscope HiPlex v2 image analysis and quantification provides a highly resolved spatial multiplexing solution to interrogate complex tissues and investigate biologically meaningful questions.

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