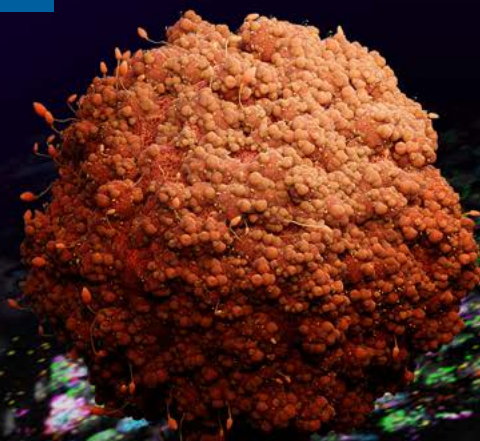


# Characterization of immune cell phenotypes through quantification of the 12-plex spatial RNAscope™ HiPlex v2 assay using the HALO® image analysis platform

Sayantani Basak<sup>1</sup>, Kim Collins<sup>2</sup>, Ching-Wei Chang<sup>1</sup>, Anushka Dikshit<sup>1</sup>

<sup>1</sup> Advanced Cell Diagnostics, Bio-Techne

<sup>2</sup> Indica Labs



## INTRODUCTION

Comprehensive spatial analysis is ideally accomplished by combining multiplexing technologies that allow simultaneous visualization of multiple target genes within the same sample with image analysis tools that can provide quantitative, single cell level data to accurately interpret gene expression within the context of the tissue.

Having been optimized for detection of target RNA in FFPE samples, the RNAscope™ HiPlex v2 assay provides a multiplexing approach ideally suited for investigating target genes in tumor tissues. We recently demonstrated the capabilities of the HiPlex v2 assay for multiplex fluorescent detection of up to 12 targets in fresh/fixed frozen and FFPE tissues<sup>1</sup>. When combined with quantitative analysis, multiplexed *in situ* RNA detection provides the capability to explore complex interactions between immune cells, tumor cells, endothelial cells, and stromal cells, thus providing an important tool for cancer research.

In this application note, we demonstrate how the HALO® image analysis platform can be employed with HiPlex v2 to (1) quantitatively assess differential expression of 12 RNA targets, (2) quantify six distinct cell phenotypes based on gene expression, and (3) analyze spatial relationships between cell phenotypes within the Tumor Microenvironment (TME). The workflow, from staining through spatial analysis, is outlined in Figure 1.

### RNAscope™ HiPlex v2-HALO FISH DIGITAL IMAGE ANALYSIS

- Quantitative cell-by-cell gene expression data on region of interest.
- Identify different phenotypes of T-cells, tumor associated macrophages and tumor cells in the TME.

## METHODS

Following the standard HiPlex v2 staining protocol using target probes for *CD3*, *CD8*, *IFNG*, *PD1*, *CD68*, *CD163*, *ARG1*, *CCL22*, *CXCL10*, *VEGFA*, *HIF1A*, and *PDL1* on a tumor microarray, images obtained from the 3 rounds of staining were registered using the RNAscope HiPlex image registration software™ from ACD, a Bio-Techne brand. Three regions of interest (ROIs) were identified in both lung and tumors\*.

For each field of view, 13 single channel tiff images were fused in HALO in order to create a single .afi fusion file containing 13 channels. Note that only one DAPI channel was utilized in the image fusion step. For all images, a rectangle annotation was created that excluded the edge image alignment variability. Image analysis was run across this Annotation Layer.

HALO v3.3.2541.301 with the FISH module v3.1.3 was used to analyze the fusion image. A single image analysis algorithm was optimized across lung and cervical cancer images. Contrast Threshold, Signal Minimum Intensity, Spot Size, Copy Intensity were optimized on a per-channel basis for each. Spot Segmentation Aggressiveness was held constant at 0.95. Cell detection parameters utilized the HALO AI™ nuclear segmentation algorithm 'AI Default'. Nuclear segmentation parameters including Minimum Nuclear Intensity, Nuclear Size, and Maximum Cytoplasm Radius were optimized across cervical and lung cancer images while Nuclear Contrast Threshold, Nuclear Segmentation Aggressiveness, Fill Nuclear Holes, and Minimum Nuclear Roundness were left at default values. Cell Localization was set to False as results on a per-cell basis were desired. FISH Scoring parameter were set at default values recommended by ACD, a Bio-Techne brand as shown in Table 1.

\*The 12-plex registered omero Tiff image generated in RNAscope HiPlex image registration software can also be used directly with HALO analysis and image fusion can be performed with the HiPlex image registration software.

Six phenotypes were defined including: *HIF1A+ PDL1+* (tumor cells), *CD3+ CD8+* (Cytotoxic T cell), *CD3+ CD8+ IFNG+ PD1+* (Activated T cells), *CD68+ CD163+* (Macrophage), *CD163+ CD68+ CCL22+* (Tumor Associated Macrophages), *VEGFA+ HIF1A+* (hypoxic cells). Single biomarker phenotypes were also desired, which HALO automatically outputs. In the Advanced analysis settings, Store Object (Cell) Data was set to True.

Proximity Analysis in HALO was performed using the **Spatial Analysis module v3.3.2541.301**. Two populations of interest were pulled into the Proximity Analysis tool from the Object Data of the FISH analysis: the *CD3+ CD8+* T-cell population and the *HIF1A+ PD-L1+* tumor cell population. The analysis was set up to count the number of *CD3+ CD8+* within 20  $\mu\text{m}$  of *HIF1A+ PD-L1+* cells. Store Object (Pair) Data was set to True to acquire Object Data on the spatial analysis. Generate Markup Image was set to True to have a Proximity Analysis view that overlays with the image data. For additional information on optimizing quantitative RNAscope image analysis, see the collaborative white paper from ACD and Indica Labs titled "Quantitative RNAscope Image Analysis Guide".

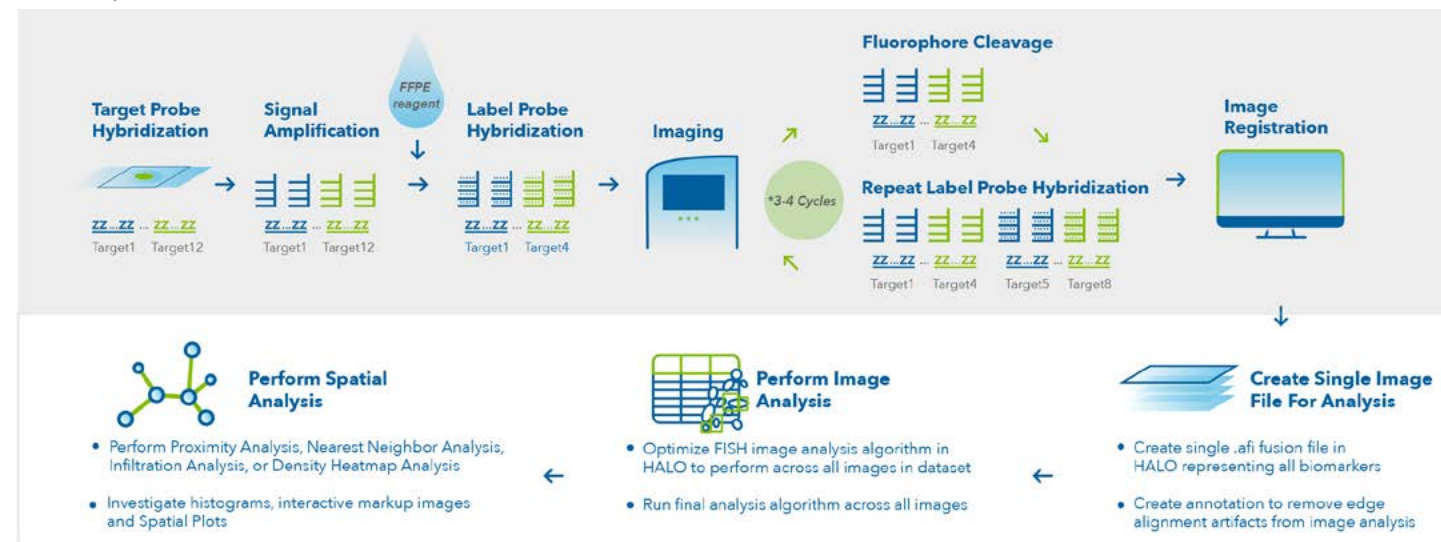
FISH SCORING PARAMETERS	
HALO	ACD
Minimum Copies/Cell	Score
1	1+
4	2+
10	3+
16	4+

TABLE 1: HALO defines RNAscope scoring according to ACD guidelines.

## RESULTS

The TME is a network of complex interactions between the tumor cells, immune cells, endothelial cells, fibroblasts, and the surrounding extracellular matrix. Immunotherapies have demonstrated therapeutic efficacy and durable responses, however some patients are either nonresponsive or develop resistance to immunotherapies. It is therefore important to determine the impact of these therapies on immune cell sub-populations and their activation states to further improve their efficacy. We have leveraged the HiPlex v2 technology to investigate spatial expression of 12 oncology

### RNAscope HiPlex AND IMAGE ANALYSIS WORKFLOW



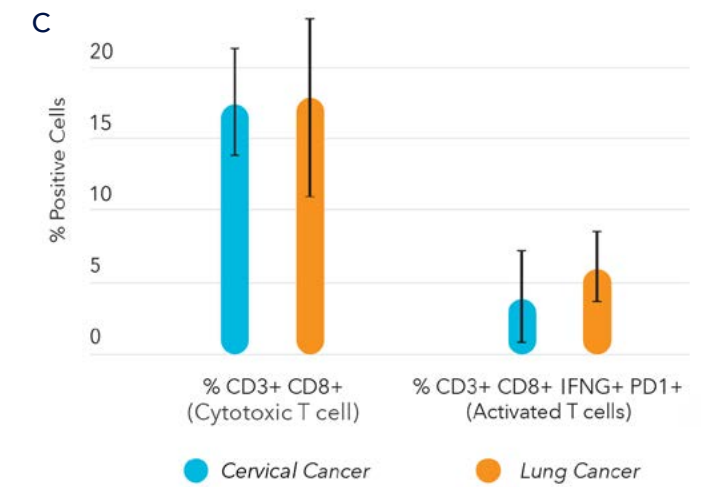
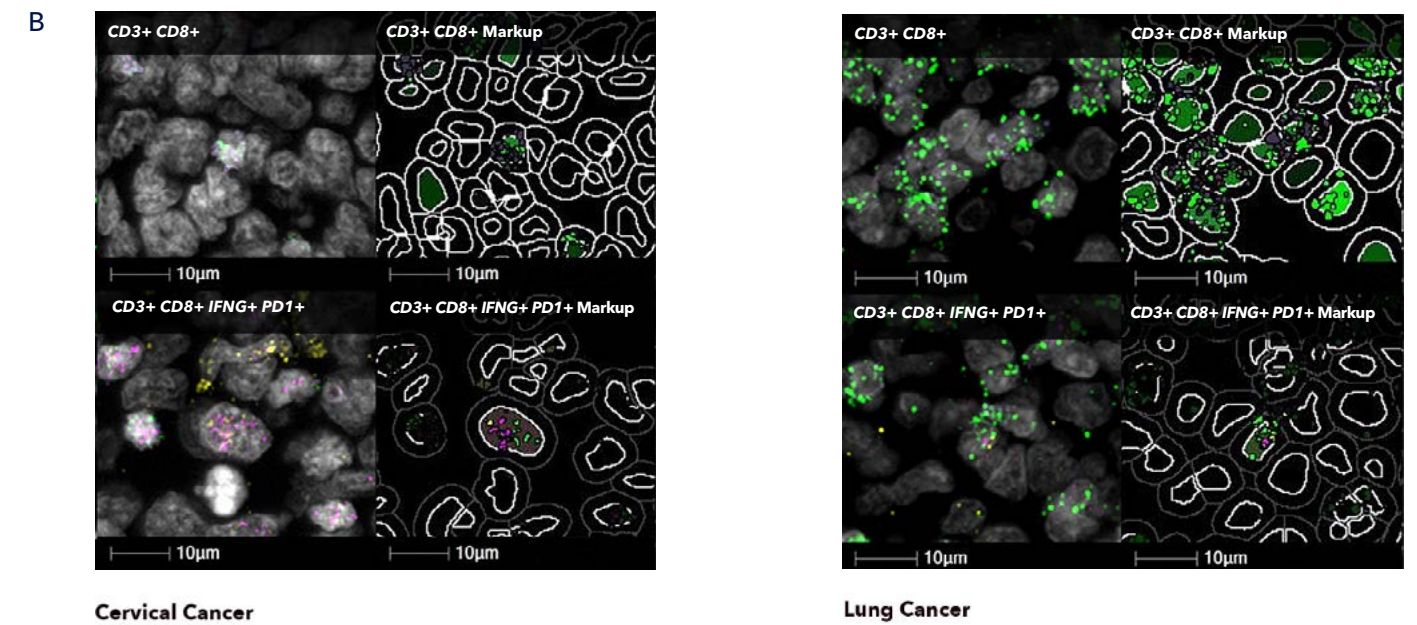
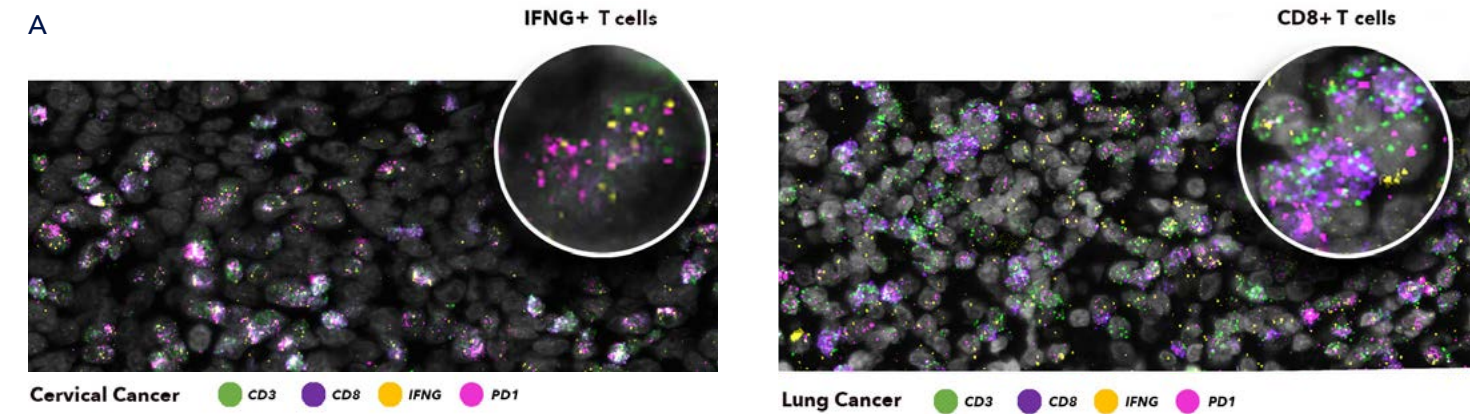
and immuno-oncology target genes, including tumor markers, immune checkpoint markers, immunosuppression markers, immune cell markers and chemokines in lung and cervical tumor tissues. Using the HALO image analysis platform, markers for T cells, macrophages and hypoxia were quantified and corresponding cell phenotypes were spatially mapped in the context of the tissue to measure proximity between immune cells and tumor cells within the TME.

We detected immune cell infiltration and identified subsets of T cells and macrophages within the TME. T cell subsets were differentiated based on target marker expression. Cytotoxic T cells were quantified using *CD3* and *CD8* expression and activated T cells by *CD3+ CD8+ PD1+ IFNG+* expression (Figure 2A). Cells with the *CD3+ CD8+* Cytotoxic T-cell phenotype and the *CD3+ CD8+ IFNG+ PD1+* Activated T-cell phenotype identified by HALO are shown in Figure 2B. Key macrophage markers *CD163*, *CD68*, *CXCL10*, *CCL22* and *ARG1* were visualized in both cervical and lung tumors (Figure 3A). Quantitative analysis indicated variable degree of infiltration of the activated T cells between the two tumors (Figure 2B and 2C). We also quantified subsets of pro- and anti-inflammatory macrophages by detecting expression of *CD68*, *CD163*, and *CCL22* (Figure 3C and 3D).

Quantitative analysis indicated higher degree of macrophage and tumor associated macrophage (TAM) infiltration in the lung tumor compared to the cervical tumors (Figure 3C). Average copy number per cell of secreted cytokines *CXCL10* and *CCL22* in cervical and lung cancer were also evaluated in these tumor samples which showed higher copy numbers in cervical tumor as compared to the lung tumor (Figure 3D).

Hypoxia has been demonstrated to reduce cell-mediated immunity and immunotherapy efficacy in most malignancies<sup>4</sup>. To investigate the immunosuppressive state of tumor cells, we detected and quantified the hypoxia markers *HIF1A* and *VEGFA* (Figure 4). Using the definitions of ACD scores provided in Table 1, the frequency of *VEGFA* or *HIF1A* classification (0+, 1+, 2+, 3+, 4+) are shown for both cervical and lung cancer samples (Figure 4B). Quantitative analysis *HIF1A* expression showed higher copy numbers in the lung tumor as compared to cervical tumor (Figure 4C). Such differences in immune signatures between tumors can be directly translated into immunotherapy success.

Figure 1. RNAscope HiPlex v2 assay, image registration and quantification workflow: Following the HiPlex assay, images were registered using the RNAscope HiPlex v2 image registration software and gene expression quantification was performed using HALO analysis.



### DIGITAL IMAGE ANALYSIS SUMMARY DATA INCLUDES:

1. Total cell count in region of interest
2. Average probe copies per cell
3. Percentage of cells for every ACD score
4. H-score for each probe
5. Co-expression data for multiplex assays

Figure 2. Detecting infiltrated T cells subsets within tumors.

A) Target probes for *CD3*, *CD8*, *IFNG* and *PD1* were used to identify different subset of T cells in cervical cancer and lung cancer.

B) 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. In the cellular images, *CD3* is shown in green, *CD8* is shown in purple, *IFNG* is shown in yellow, *PD1* is shown in magenta, and DAPI is shown in gray. 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.

C) Quantification of *CD3+ CD8+* cytotoxic T cells, and *CD3+ CD8+ IFNG+ PD1+* activated T cells using the HALO FISH module.

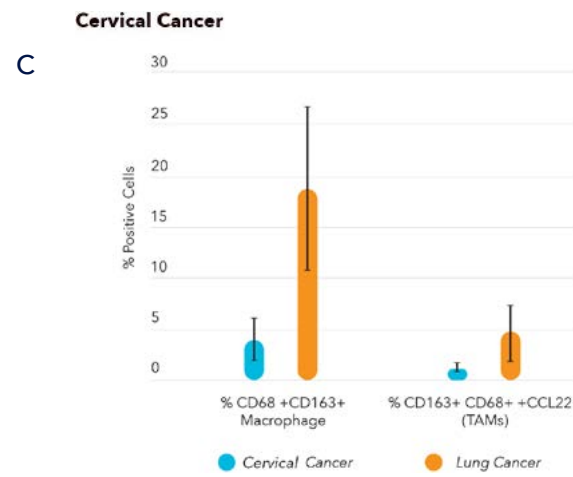
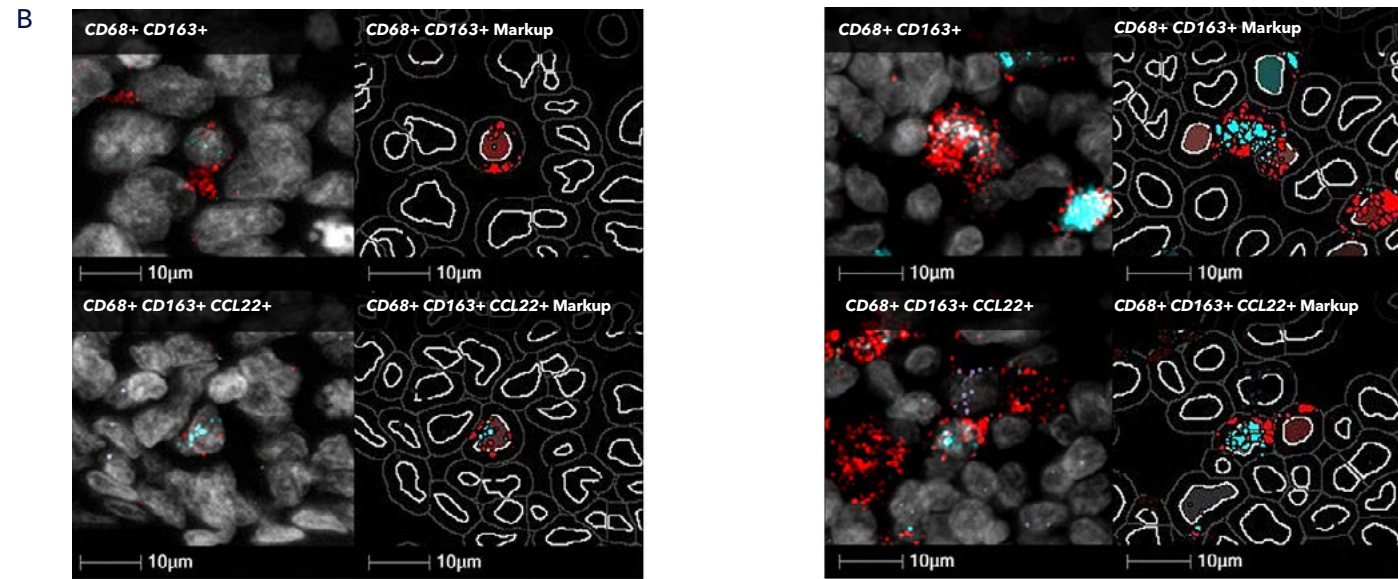
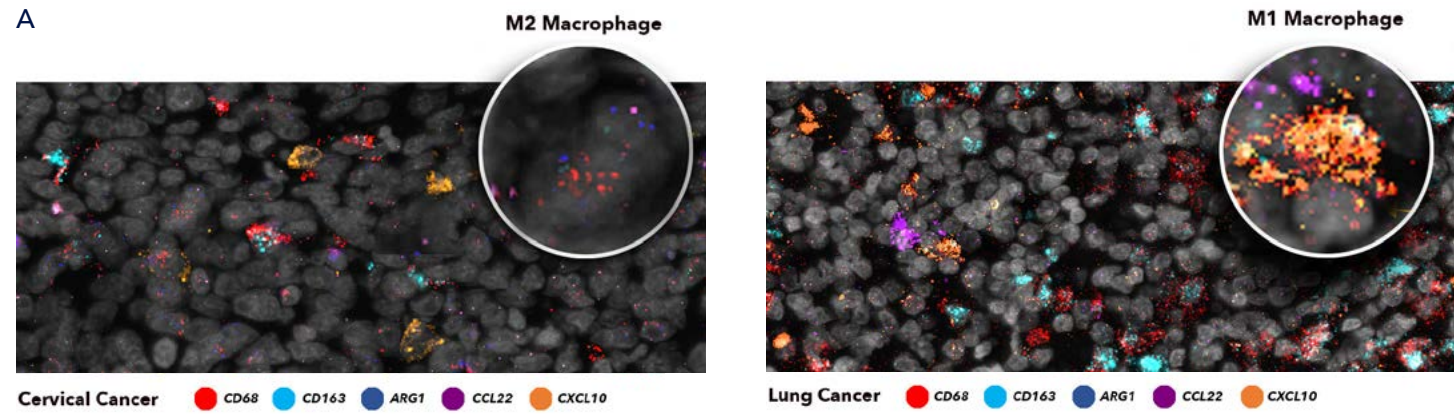


Figure 3: Detecting tumor associated macrophages markers (TAMS), chemokines and cytokines within tumors.

A) 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.

B) Representative *CD68+ CD163+* macrophages and *CD68+ CD163+ CCL22+* TAM cells and the corresponding HALO markup is shown for each phenotype for cervical and lung cancer samples. In the cellular images, *CD68* is shown in red, *CD163* is shown in cyan, *CCL22* is shown in light purple, and DAPI is shown in gray. 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.

C) Quantification of *CD68+ CD163+* (total macrophages) and TAMs using HALO FISH module.

D) Average copy number per cell of secreted cytokines *CXCL10* and *CCL22* in cervical and lung cancer.

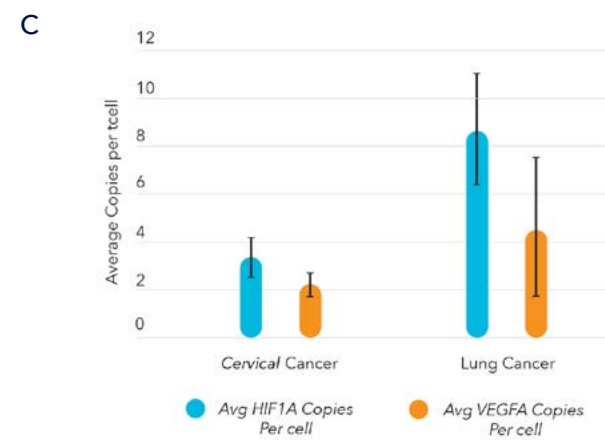
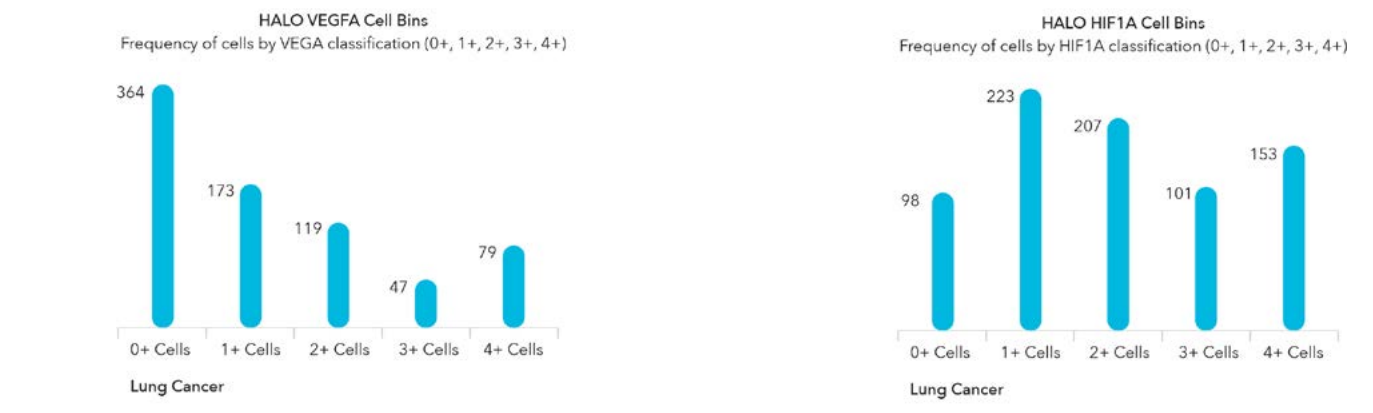
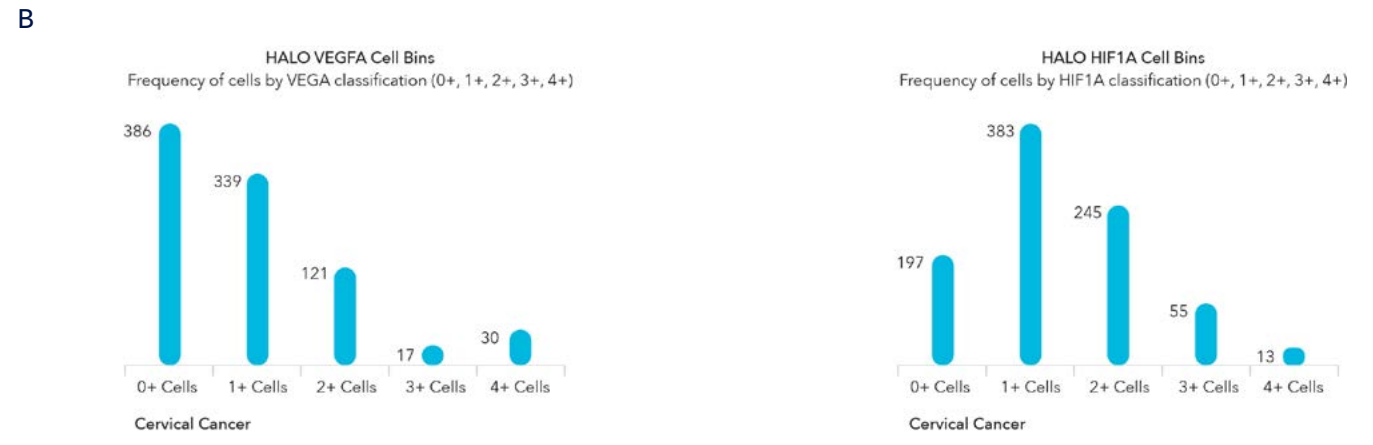
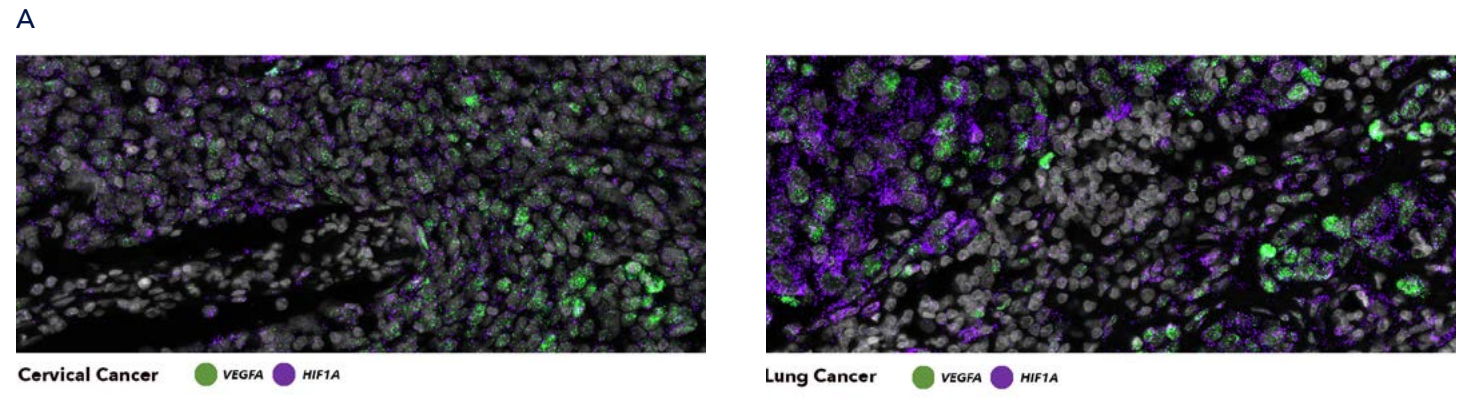


Figure 4: Detecting hypoxia markers in the tumor microenvironment. Level of hypoxia was assessed in different tumor samples.

A) Detecting the expression of *HIF1A* and *VEGFA*.

B) HALO analysis histogram shows frequency of cells according to *VEGFA* or *HIF1A* classification across ACD scores for both cervical and lung cancer samples. Each histogram represents analysis of a single image.

C) Quantification of *HIF1A* and *VEGFA* copy numbers per cell using HALO FISH module. This analysis was performed on 2 ROIs for lung cancer and 3 ROIs for cervical cancer.

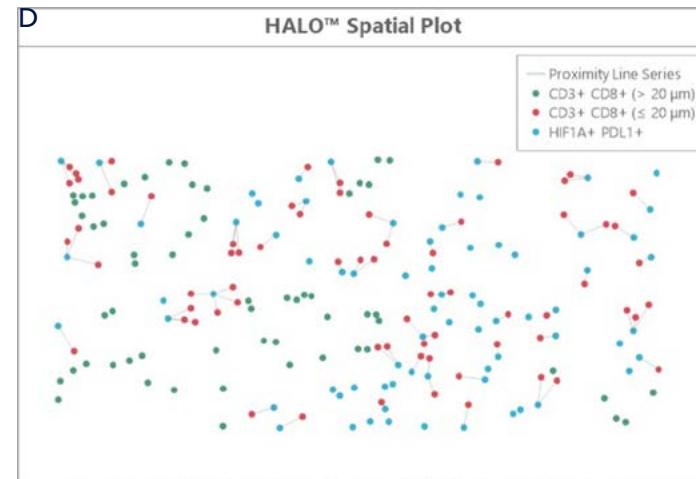
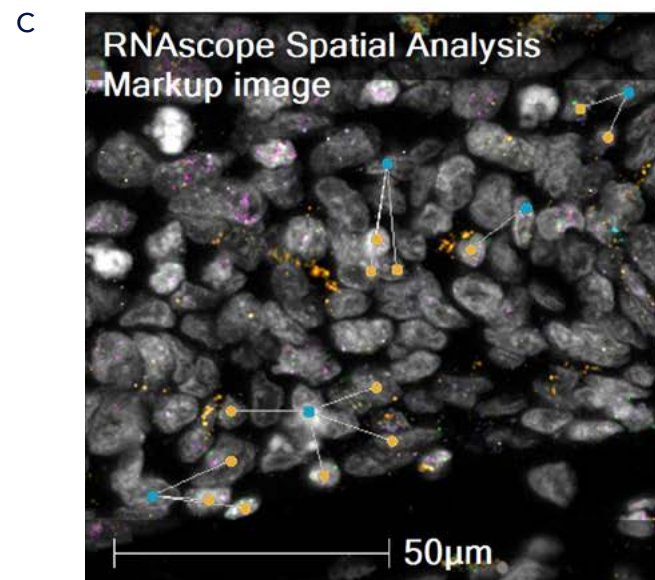
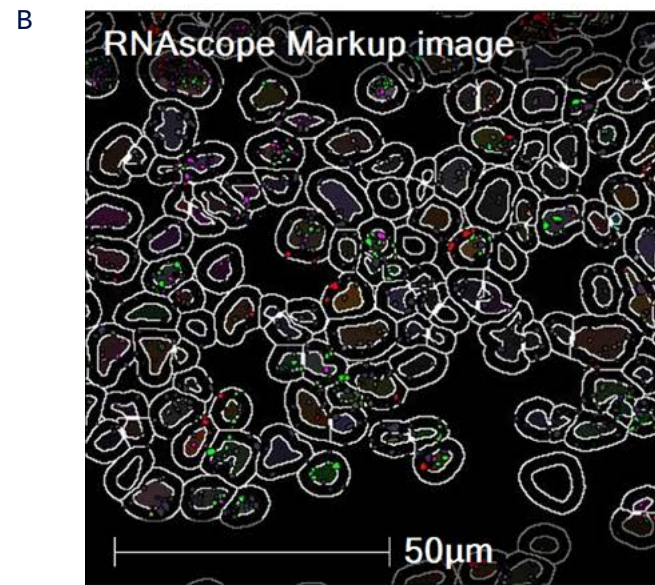
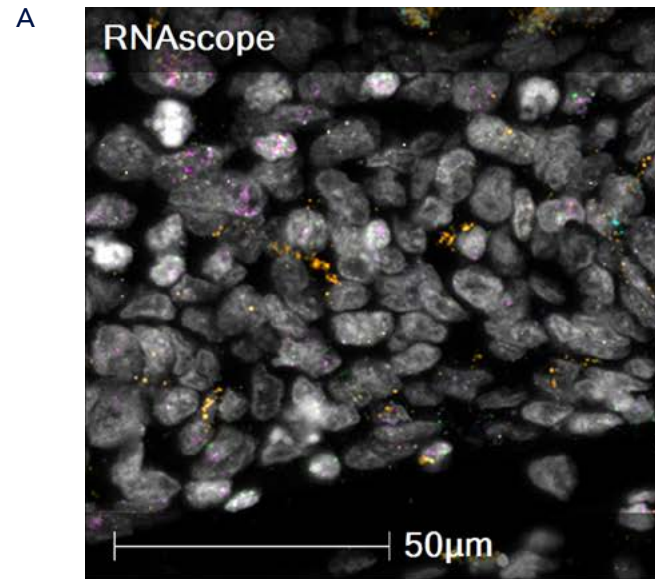


Figure 5. Proximity Analysis of CD3+ CD8+ T cells within 20 µm of HIF1A+ PD-L1+ tumor cells in cervical cancer tissue.

A) ROI from a 12-plex RNAscope image.

B) FISH analysis is performed 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. An interactive HALO markup image is shown. *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. 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.

### EXPLORE SPATIAL RELATIONSHIPS BETWEEN IMMUNE CELLS BY PERFORMING HALO PROXIMITY ANALYSIS FOR RNAscope SIGNALS

The [Spatial Analysis](#) module of HALO includes tools that can perform Proximity Analysis, Nearest Neighbor Analysis, Infiltration Analysis, and Density Heatmap Analysis to further explore spatial relationships between cells within the TME. Proximity Analysis calculates the number of cells within a certain distance of another type of cell. A Proximity Analysis to determine the number of *CD3+ CD8+* cells (cytotoxic T-cells) within 20 µm of *HIF1A+ PD-L1+* (tumor cells) in cervical cancer tissue was performed (Figure 5). First, FISH analysis is performed on the 12-plex assay which yields Object Data that includes positional information on each cell as well as phenotypic information. Any phenotype of interest represented in the Object Data can be used in the Proximity Analysis. HALO offers multiple outputs for Proximity Analysis including an interactive markup image (Figure 5C) and a Spatial Plot (Figure 5D). The Spatial Plot result shows cytotoxic T-cells within 20 µm of a tumor cell as red, and T-cells that are 20 µm away from a tumor cell as green, indicating T-cell recruitment at the tumor site (Figure 5D).

### SUMMARY

In this document we have demonstrated the capability of RNAscope HiPlex v2 quantification using HALO image analysis. Here we have shown that using the RNAscope HiPlex image registration software, regions of interest can be aligned across 3 rounds of imaging and a single image can be created in HALO for quantitative image analysis. Using the FISH module of HALO, we demonstrate quantitative analysis of 12 target RNA markers on different tumor tissues to characterize specific regions of interest. Previously, using the RNAscope HiPlex v2 assay we were able to successfully profile the TME to identify immune cell sub-types and assess the inflammatory signature within each tumor sample<sup>1</sup>. While the images shown in this document are representative fields of view, it is important to note that optimized ISH and FISH analysis settings can be applied to whole tissue sections and across batches of images using the HALO platform thus offering a means to generate quantitative data in a high throughput manner. In order to perform whole-slide image analysis using the RNAscope HiPlex v2 assay, image registration and alignment would need to be performed in HALO using the [Serial Registration Analysis Add-on](#).

We have further demonstrated a workflow where spatial analysis can be performed on FISH Object Data in HALO. Here we show a proximity analysis examining the proximity of T cells and tumor cells in cervical cancer, since in many other cancer types, a positive correlation between survival and *CD8+* T-cell density in the tumor core is reported. Although this Application Note demonstrates spatial analysis on ROIs, when performing spatial analysis examining rare phenotypes, it is recommended to perform this workflow using whole slide images.

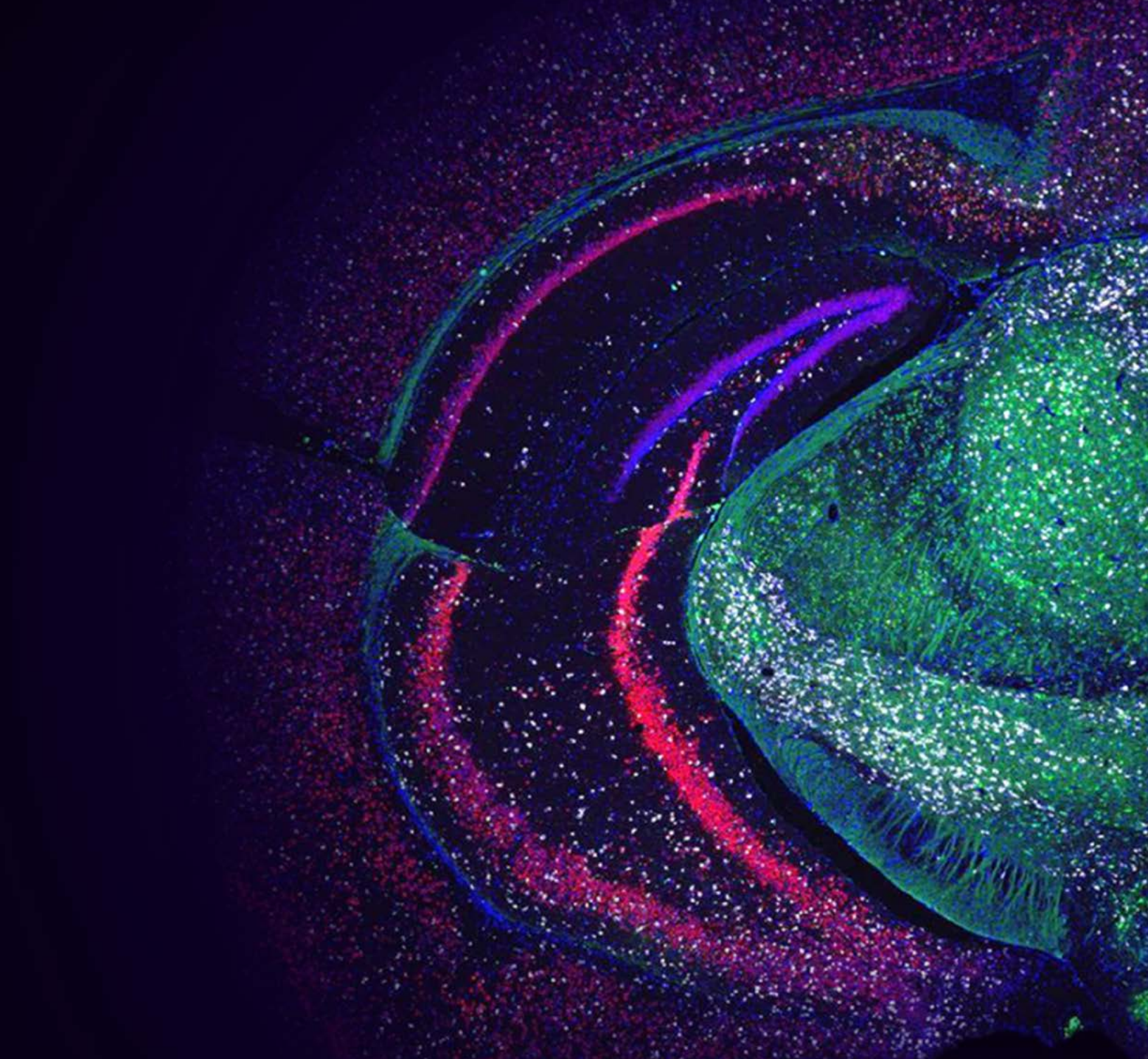
In addition to Proximity Analysis, the Spatial Analysis module of HALO enables Infiltration Analysis. With Infiltration Analysis, an invasive margin is defined automatically or can be drawn manually, and then the number of cells are determined in a user-defined range of distances inside, outside, or inside and outside the invasive margin. Infiltration Analyses are clinically relevant as understanding the extent of T cell infiltration in a tumor can be prognostic. In colon cancer, high infiltration of T cells correlates with a high Immunoscore, which indicates a low risk of relapse. Conversely, a low infiltration of T cells yields a low Immunoscore that correlates with a high risk of relapse. The Immunoscore method is currently being investigated in other cancers for prognostic value<sup>3</sup>.

In conclusion, 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.

### REFERENCES

1. Profiling of Immune Cell Gene Signatures in The Tumor Microenvironment. Anushka Dikshit, Sayantani Basak, Ming Yu, HaYeun Ji, Bingqing Zhang.
2. Quantitative RNAscope Image Analysis Guide, a collaborative white paper from Indica Labs and ACD, a Bio-technie brand.
3. The immune contexture and Immunoscore in cancer prognosis and therapeutic efficacy. Bruni D et. al., Nature Reviews Cancer volume 20, (2020)
4. Improving Cancer Immunotherapy by Targeting the Hypoxic Tumor Microenvironment: New Opportunities and Challenges. Noman MZ et. al., Cells. (2019)

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