STIC, 2024, Poster #78

Spatial multi-omics analysis of the tumor microenvironment utilizing high-plex HCR Gold RNA-FISH and high-resolution immunofluorescence

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Introduction

The tumor-immune microenvironment (TiME) is characterized by significant proteomic and transcriptomic heterogeneity. Spatial multi-omics combines high-plex spatial proteomics and transcriptomics to effectively address TiME complexity in tumor tissues. Hybridization Chain Reaction RNA fluorescence *in situ* hybridization (HCR™ RNA-FISH) assays employ conditional nucleic acid self-assembly to achieve enzyme-free, quantitative signal amplification. HCR™ assays are natively compatible with multiplex immunofluorescence (mIF) due to protease-free sample preparation and therefore enable RNA and protein co-detection on the same tissue sample.

Methods

We developed an iterative multiomic assay for the CellScape™ Precise Spatial Proteomics platform that enabled interrogation of 12 RNA targets via HCR™ Gold RNA FISH and 30 protein markers via mIF on the same formalin-fixed paraffin-embedded (FFPE) tissue section. HCR™ Gold RNA-FISH target probes were added to the tissue after dewaxing and antigen retrieval. The sample was then enclosed in a CellScape™ Whole Slide Imaging Chamber to enable rounds of on-instrument staining, imaging, and signal removal via EpicIF™ (Enhanced photobleaching in cyclic immunofluorescence) technology. Detection of HCR™ Gold probes was achieved by addition of target-specific fluorescently labeled HCR™ Gold amplifiers for signal generation. The HCR™ assay was followed by 30-plex mIF. Some antibodies were directed against protein targets corresponding to RNA targets interrogated with RNA-FISH to show congruence of the two assays. RNA-FISH and mIF image data were processed and integrated with a custom Python-based image analysis pipeline.

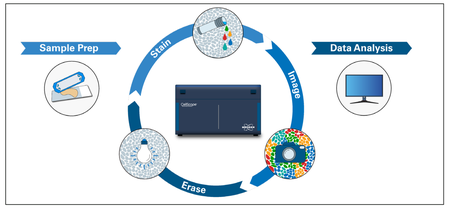

Figure 1. The EpicIF workflow on the CellScape platform uses cycles of staining, imaging, and signal removal to detect biomarkers with spatial context at single-cell resolution. Signal removal facilitated by filtered photobleaching and EpicIF™ Solution is safe, gentle, and effective.

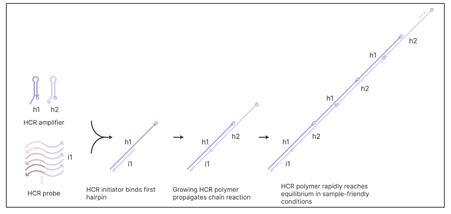

Figure 2. HCR Gold RNA-FISH workflow.

EpicIF technology enables same tissue mIF with HCR Gold RNA-FISH

Table 1. Assay layout by cycle and channel. RNA-FISH was performed first (cycle 1-3), followed by DNA and mIF stains. Complete signal removal between cycles was accomplished with EpicIF Solution and filtered photobleaching.

Cycle	488	532	594	647	Cycle	488	532	594	647
1 (RNA)	CD3E	CD274	TNFA	TIGIT	7	CD11c	Ki-67	CD8a	CD4
2 (RNA)	KRT19	PDCD1	FOXP3	LAG3	8	Gran B	CD34	CD68	CD138
3 (RNA)	MALAT1	CD8A	IL6	CTLA4	9	LAG-3	CD31	CK19	ER
4	DNA				10	CD16		CD14	CD20
5	CD3	CD45	CD274	HLA-DR	11	HER2		FOXP3	CD66b
6	CD279	Vimentin	CD163	CD123	12	p53	SMA	B-Catenin	PR

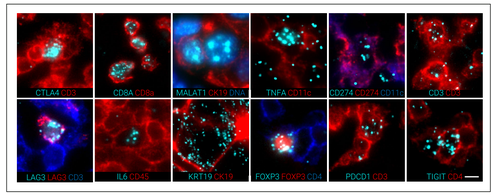

Figure 3. The subcellular resolution of the CellScape platform allows precise localization and quantification of RNA-FISH signals. Shown are example images for each interrogated RNA target. The RNA-FISH signal is shown in cyan; reference antibody stains are shown in red and blue. The scalebar represents 5 µm and applies to all images.

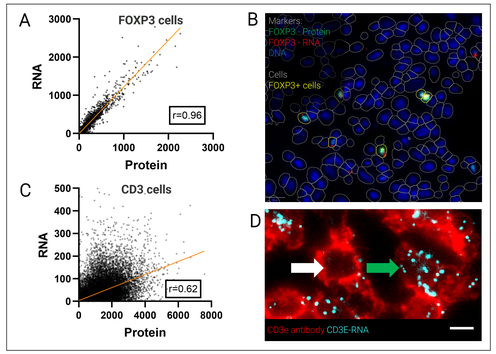

Figure 4. Depending on the target, correlation between RNA and antibody signal intensity can vary. **A.** Plot shows high correlation for FOXP3. **B.** Example image of segmented FOXP3+ cells. **C.** In CD3 cells, the correlation between CD3 RNA and protein signals is lower. **D.** Example image of CD3 cells. White arrow marks cell with high protein and low RNA; the green arrows marks low/intermediate protein and high RNA.

The CellScape high-plex multiomic spatial assay (mIF + RNA-FISH) reveals intra-tumoral and TiME heterogeneity

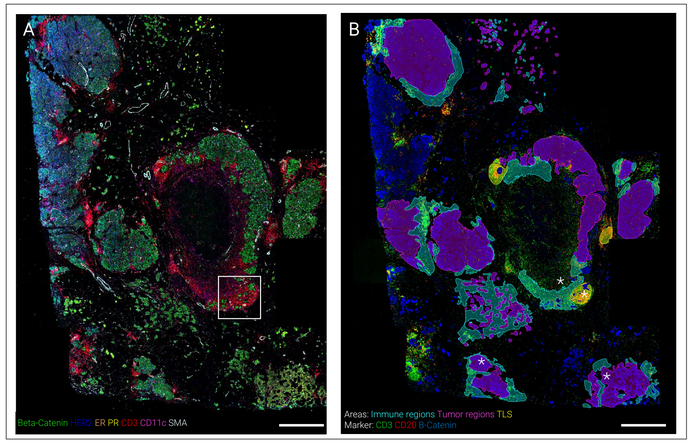

Figure 5. Multiplex immunofluorescence staining allows segmentation of the tumor and TiME into multiple regions based on biomarker expression. **A.** Overview of a Breast Cancer (BC) tissue (Invasive Ductal Carcinoma). Areas with differential expression of classical BC markers HER2, ER and PR, as well as multiple lymphocyte rich clusters are visible. These features permit regional segmentation and mapping of the TiME as in **B**. The region marked with a white square contains a tertiary lymphoid structure (TLS) which is shown in more detail in **Fig. 6**. Asterisks mark regions shown in Fig. 8. The scalebar represents 800 µm and applies to both panels.

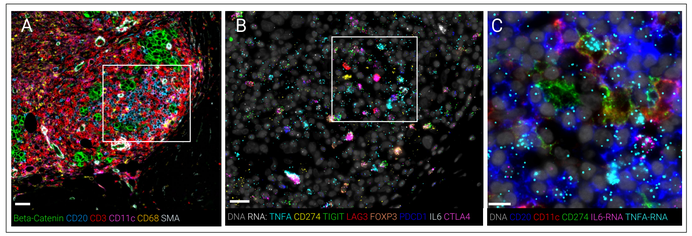

Figure 6. The multiomic assay allows deeper insight into immune regulatory processes in the TiME. **A.** Shows intra-tumoral tertiary lymphoid structure with lymphocytes and follicular dendritic cells (FDC). The marked region is shown again in **B.** RNA FISH staining allows the additional detection of immune regulatory factors in the TLS that are difficult to detect by antibody staining (e.g. secreted cytokines like IL6 and TNFA). Marked region is further amplified in **C.** Expression of TNFA is elevated in B-cells, FDCs and unidentified cell type in the center region of the TLS. Scalebars represent in **A.** 50 µm, **B.** 20 µm, **C.** 10 µm.

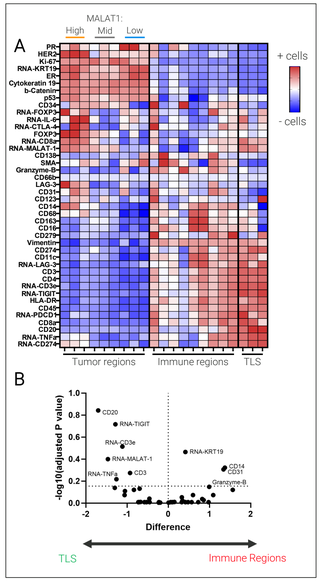

Figure 7. Cell segmentation and signal quantification shows differential RNA and protein staining across the tissue. **A.** Heatmap illustrating overview of protein and RNA marker distribution across distinct microenvironmental regions shown in **Fig. 5** including tumor, immune and TLS. Color intensity denotes cell density per identified area. **B.** The TLS regions show significantly higher expression of lymphocyte markers, the cytokine TNFA, and MALAT1 compared to other immune regions.

RNA-FISH shows differential expression of non-coding RNA

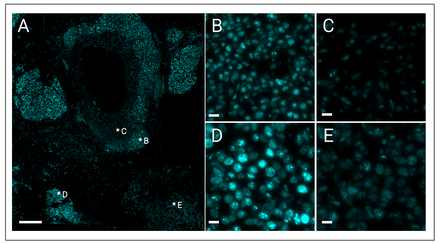

Figure 8. Differential expression of long non-coding RNA MALAT1 in the tumor and TiME indicates potential role in tumor progression and immune regulation. **A.** Shows an overview of MALAT1 expression in the tissue. Scalebar = 500 µm. Regions shown in **B-E** are marked with asterisks. **B.** MALAT1-high immune region in TLS. **C.** MALAT1-low immune region. **D.** MALAT1-high tumor region. **E.** MALAT1-low tumor region. Scalebars = 10 µm.

Conclusions

- EpicIF™ technology enables iterative spatial transcriptomic and proteomic co-detection on the same sample.
- Protease-free HCR™ Gold RNA-FISH is fully compatible with mIF assay integration on the CellScape™ platform.
- Combined multiomic readouts allow more precise characterization of immune processes like TLS formation in the TiME.
- RNA-FISH enables investigation of cytokine expression within specific cell types as well as non-coding RNAs like MALAT1 in tumor progression and immune response.

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