SITC 2023 Abstract #1503

Optimization of an Integrated MultiOmyx-RNAscope hyperplex assay for co-detection and characterization of multiple protein and RNA biomarkers within the tumor microenvironment (TME)

Courtney Todorov · Sandra Lam · Harry Nunns · Kevin Gallagher · Juliana Wortman · Eric Leones · Elaine Yeung · Flora Sahafi · Erinn A. Parnell · Qingyan Au

NeoGenomics Laboratories, Aliso Viejo, CA

Background: Spatial analysis of protein or gene expression is vital to understand the distribution, phenotypes, and interactions between cells within TME. Traditionally, multiplexed spatial analysis has been performed using methods to detect either protein or RNA separately. Combining spatial analysis of protein-RNA on a single specimen is a powerful method to identify the cellular source of secreted proteins, characterize the cytokine signature, study gene expression in specific cell types as defined by protein biomarkers, or map the distribution of CAR-T+ cells within the TME. We have previously demonstrated validation of an integrated workflow to co-detect RNA and protein in a single FFPE slide using the MultiOmyx™ (NeoGenomic Laboratories, Inc) and RNAscope™ (Bio-Techne) platforms. MultiOmyx is a proprietary immunofluorescence (IF) platform for the visualization and characterization of up to 60 protein biomarkers in a single FFPE section. RNAscope Multiplex is a highly sensitive fluorescent in-situ hybridization (ISH) assay that can detect up to 3 RNA markers in a single FFPE section. A unique feature of the Integrated MultiOmyx-RNAscope assay is the presence of a protease pretreatment step, which is required for RNAscope ISH staining but can damage proteins and consequently interfere with downstream antibody-antigen binding.

We previously demonstrated robustness of an Integrated assay to characterize infiltrating lymphocytes within TME. However, subsequent testing of additional protein biomarkers with the Integrated assay has shown some incompatibility or suboptimal signal to noise (S/N). Individual optimization steps to improve protein biomarker S/N and compatibility are time consuming and can be limited by antibody clone availability. Therefore, we decided to globally optimize the Integrated MultiOmyx-RNAscope assay to improve overall protein biomarker performance.

Methods: Optimization of both antigen retrieval and protease pretreatment steps was performed to improve protein biomarker performance. A validation of the optimized Integrated MultiOmyx-RNAscope assay was then completed using a 2-plex ISH 17-plex IF biomarker panel on FFPE human colorectal cancer (CRC) samples. Expression of each ISH and protein biomarker was quantified using NeoLYTX™, the proprietary MultiOmyx Analytics pipeline and inter/intra run coefficient of variation (CV) were calculated for the precision assessment.

Results The optimized Integrated assay demonstrated improved protein biomarker staining/compatibility without compromising RNA ISH signal. Additionally, all markers evaluated showed highly reproducible results and passed successful criteria for precision evaluation. These results therefore demonstrate a highly robust assay with even improved performance observed for some markers previously evaluated.

Conclusions: Therefore, we successfully optimized the Integrated MultiOmyx-RNAscope assay for co-detection of protein/RNA in single specimen thereby improving assay development turnaround and protein biomarkers performance/compatibility.

Integrated MultiOmyx-RNAscope Assay Workflow and 2plx-ISH 17plx-IF Validation Biomarker Panel

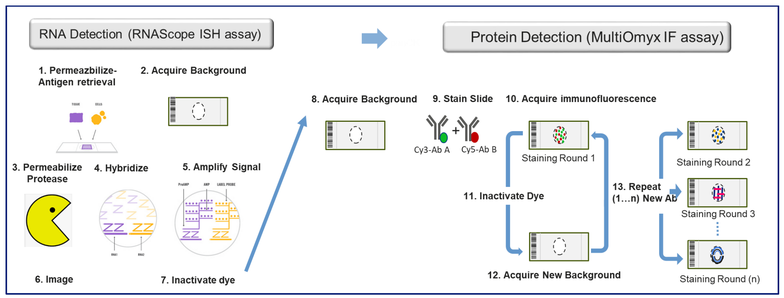


Figure 1. Integrated MultiOmyx-RNAscope Assay Workflow. The integrated workflow combines RNAscope ISH and MultiOmyx multiplexing IF staining protocols. Slides were first cleared and undergo pretreatment, which includes both heat activated epitope retrieval and protease steps. Then RNAscope ISH probe hybridization and staining was performed before proceeding to IF multiplexing. For each round of IF staining, conjugated fluorescent antibodies were applied to the slide, followed by imaging acquisition of stained slides. The dye was erased, enabling a second round of staining with another pair of fluorescent antibodies. The optimized Integrated MultiOmyx-RNAscope assay version 2 (V2) used for this study differs from the previous Integrated assay version 1 (V1) in pretreatment conditions used and not overall workflow.

R&R Panel Biomarkers	
IFNg-ISH	CXCL10-ISH
alpha-SMA	CD163
CD3	FOXP3
CD4	Granzyme B
CD8	HLA-ABC
CD11b	HLA-DR
CD11c	NKp46
CD20	PanCK
CD68	PD-1
–	PD-L1

Validation of an Integrated MulitOmyx-RNAscope Assay for co-detection of RNA/protein

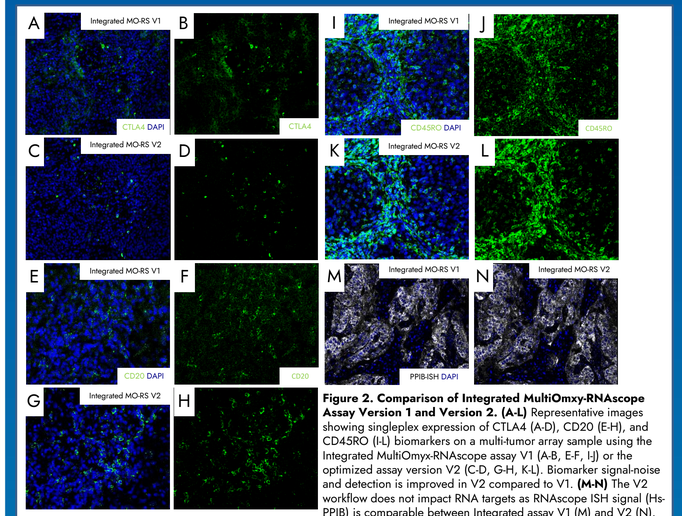


Figure 2. Comparison of Integrated MultiOmxy-RNAscope Assay Version 1 and Version 2. (A-L) Representative images showing singleplex expression of CTLA4 (A-D), CD20 (E-H), and CD45RO (I-L) biomarkers on a multi-tumor array sample using the Integrated MultiOmyx-RNAscope assay V1 (A-B, E-F, I-J) or the optimized assay version V2 (C-D, G-H, K-L). Biomarker signal-noise and detection is improved in V2 compared to V1. **(M-N)** The V2 workflow does not impact RNA targets as RNAscope ISH signal (Hs-PPIB) is comparable between Integrated assay V1 (M) and V2 (N).

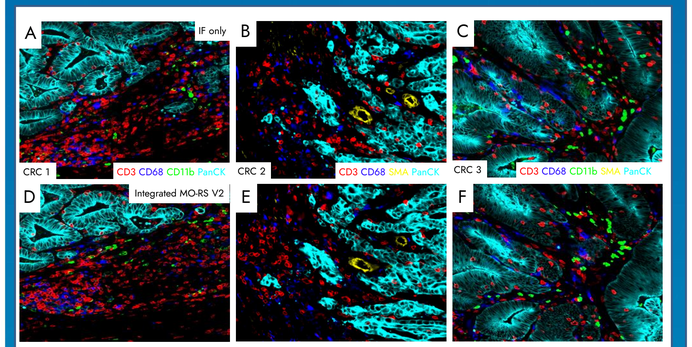
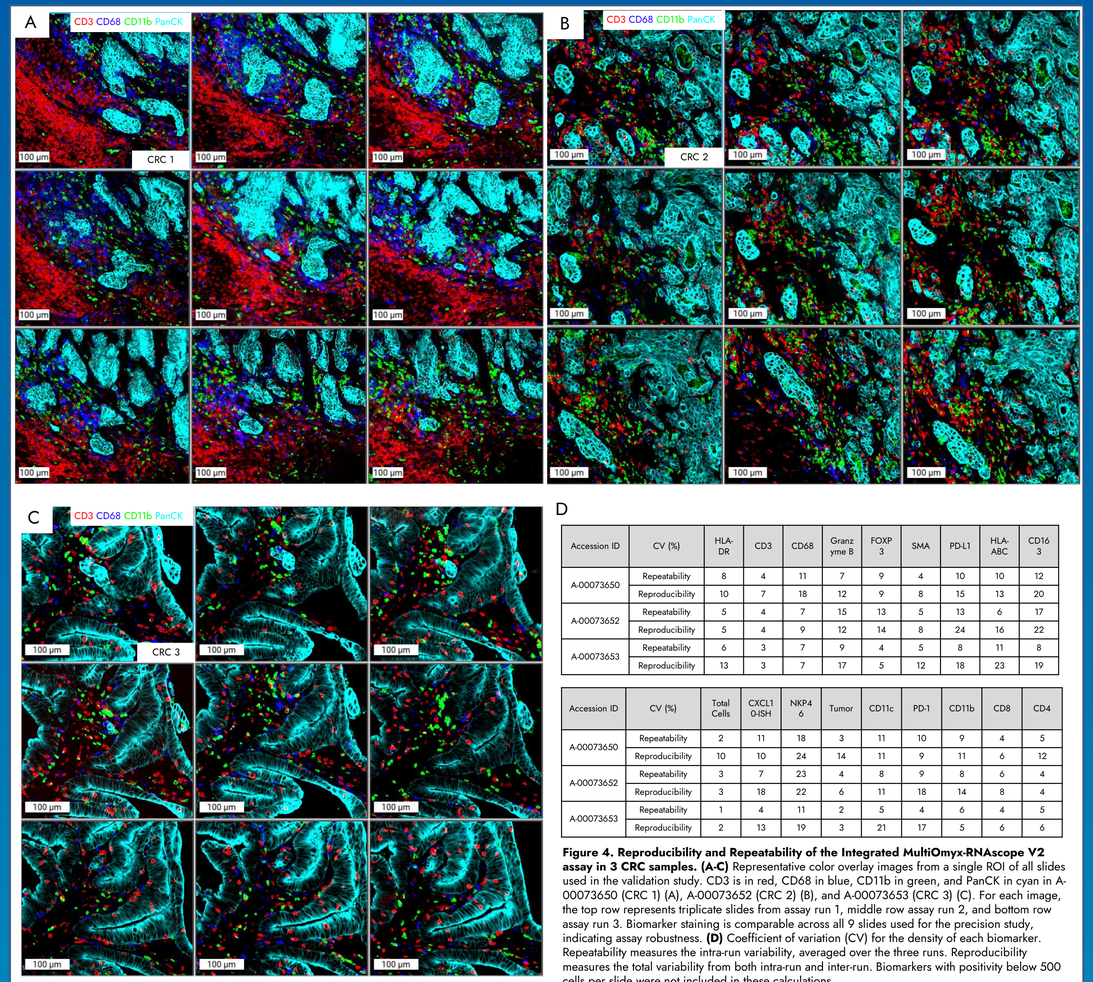


Figure 3. Comparison of biomarker staining with the MultiOmyx IF-only or Integrated MultiOmyx-RNAscope V2 workflow in CRC samples. Representative color overlay images showing biomarker expression in three CRC samples stained using IF only (A-C) or Integrated MultiOmyx-RNAscope V2 (D-F) workflow. In all 3 CRC samples evaluated, biomarker staining is comparable between the workflows, demonstrating biomarker specificity with the MultiOmyx-RNAscope V2 assay.



D

Accession ID	CV (%)	HLA-DR	CD3	CD68	Granzyme B	FOXP3	SMA	PD-L1	HLA-ABC	CD163
A-00073650	Repeatability	8	4	11	7	9	4	10	10	12
	Reproducibility	10	7	18	12	9	8	15	13	20
A-00073652	Repeatability	5	4	7	15	13	5	13	6	17
	Reproducibility	5	4	9	12	14	8	24	16	22
A-00073653	Repeatability	6	3	7	9	4	5	8	11	8
	Reproducibility	13	3	7	17	5	12	18	23	19

Accession ID	CV (%)	Total Cells	CXCL10-ISH	NKP46	Tumor	CD11c	PD-1	CD11b	CD8	CD4
A-00073650	Repeatability	2	11	18	3	11	10	9	4	5
	Reproducibility	10	10	24	14	11	9	11	6	12
A-00073652	Repeatability	3	7	23	4	8	9	8	6	4
	Reproducibility	3	18	22	6	11	18	14	8	4
A-00073653	Repeatability	1	4	11	2	5	4	6	4	5
	Reproducibility	2	13	19	3	21	17	5	6	6

Figure 4. Reproducibility and Repeatability of the Integrated MultiOmyx-RNAscope V2 assay in 3 CRC samples. (A-C) Representative color overlay images from a single ROI of all slides used in the validation study. CD3 is in red, CD68 in blue, CD11b in green, and PanCK in cyan in A-00073650 (CRC 1) (A), A-00073652 (CRC 2) (B), and A-00073653 (CRC 3) (C). For each image, the top row represents triplicate slides from assay run 1, middle row assay run 2, and bottom row assay run 3. Biomarker staining is comparable across all 9 slides used for the precision study, indicating assay robustness. **(D)** Coefficient of variation (CV) for the density of each biomarker. Repeatability measures the intra-run variability, averaged over the three runs. Reproducibility measures the total variability from both intra-run and inter-run. Biomarkers with positivity below 500 cells per slide were not included in these calculations.

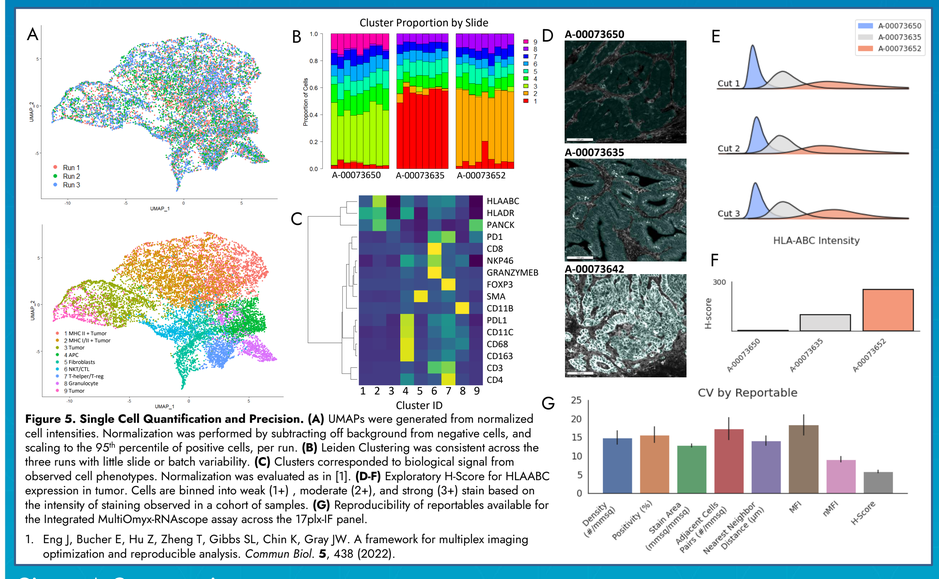


Figure 5. Single Cell Quantification and Precision. (A) UMAPs were generated from normalized cell intensities. Normalization was performed by subtracting off background from negative cells, and scaling to the 95th percentile of positive cells, per run. **(B)** Leiden Clustering was consistent across the three runs with little slide or batch variability. **(C)** Clusters corresponded to biological signal from observed cell phenotypes. Normalization was evaluated as in [1]. **(D-F)** Exploratory H-Score for HLAABC expression in tumor. Cells are binned into weak (1+) , moderate (2+), and strong (3+) stain based on the intensity of staining observed in a cohort of samples. **(G)** Reproducibility of reportables available for the Integrated MultiOmyx-RNAscope assay across the 17plx-IF panel.

1. Eng J, Bucher E, Hu Z, Zheng T, Gibbs SL, Chin K, Gray JW. A framework for multiplex imaging optimization and reproducible analysis. *Commun Biol.* **5**, 438 (2022).

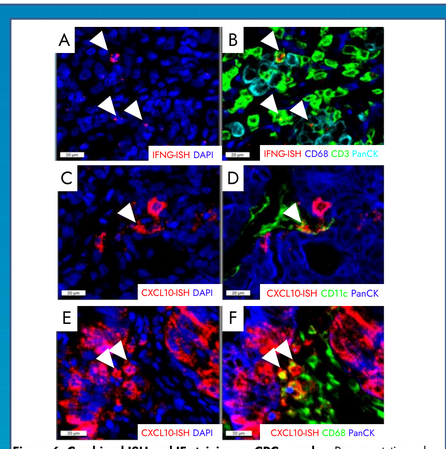


Figure 6. Combined ISH and IF staining on CRC samples. Representative color overlay images showing co-expression of ISH markers in different CRC TME populations. **(A-B)** IFNG-ISH in red and DAPI in blue (A) and IFNG-ISH in red, CD3 in green, and PanCK in cyan (B). White arrow shows IFNG-ISH expression overlay with T cell marker CD3. **(C-D)** CXCL10-ISH in red and DAPI in blue (C) and CXCL10-ISH in red, CD11c in green, and PanCK in blue (D). CXCL10-ISH+CD11b+ in yellow (white arrow) and PanCK in magenta. **(E-F)** CXCL10-ISH in red and DAPI in blue (E) and CXCL10-ISH in red, CD68 in green, and PanCK in blue (F). CXCL10-ISH+ CD68+ cells in yellow (white arrows) and CXCL10-ISH+ tumor in magenta.

Summary

- Integrated MultiOmyx-RNAscope assay V2 shows improved biomarker signal/noise and detection.
- Precision study of V2 assay in 3 CRC samples demonstrates robustness of biomarkers analyzed.
- Co-detection of RNA and protein shows correlation of cytokine RNA expression in different populations of the TME.
- The optimized Integrated MultiOmyx-RNAscope V2 assay is a robust and sensitive planform for co-detection of RNA/protein in a single FFPE sample.

Qingyan.Au@neogenomics.com