# Integrated Analysis of MicroRNA, mRNA, and Protein Expression Utilizing MultiOmyx™ and NanoString™ from Formalin-Fixed Paraffin-Embedded Lung, Head and Neck, Breast, and Melanoma Tumors

#1469

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## Background

Cancer is characterized as a loss of normal cellular regulation, due to accumulation of mutations and disruption of complex biological pathways. MicroRNAs (miRNAs) regulation of co-stimulatory and immune checkpoint pathways have been implicated as one of the potential mechanisms for cancer evasion in immuno-oncology. It is estimated that 30% of all mRNA expression may be regulated by miRNAs, and some are either oncogenic or tumor suppressive. Complexity of miRNA regulation highlights the need for integrated assays, providing direct correlation between miRNA and mRNA, and protein expression.

MultiOmyx, a novel hyperplexed multi "omic" technology, enables visualization and characterization of multiple biomarkers across multiple assays on **a single 4µm tissue section**. MultiOmyx protein immunofluorescence (IF) assays utilize a pair of directly conjugated Cyanine dye-labeled (Cy3, Cy5) antibodies per round of staining. Each round of staining is imaged and followed by novel dye inactivation chemistry, enabling repeated rounds of staining and deactivation for up to 60 protein biomarkers. In this study, MultiOmyx hyperplexed IF assay was utilized to measure CD3, CD4, CD8, CD16, CD56, GRANZYME B , FOXP3, ICOS, OX40, OX40L, PD1, PD-L1 and HLA-DR protein expression from a single 4 µm FFPE section. From an adjacent 10 µm section, NanoStringTM nCounter PanCancer Immune Profiling Panel and Human v3 miRNA expression panel were utilized to comprehensively profile the expression of 770 mRNA and 800 miRNA.

Integrating MultiOmyx and NanoString technologies, the current study measured miRNA, mRNA, and protein expression in lung, head and neck, breast, and melanoma samples. For each indication, three samples were selected from a larger sample set, based on high protein expression of lymphocytes and macrophage markers (CD3, CD4, CD8, CD56, CD16), co-stimulator markers (ICOS, OX40), and immune checkpoint markers (PD1, PD-L1).

## Overview of Assay Workflow

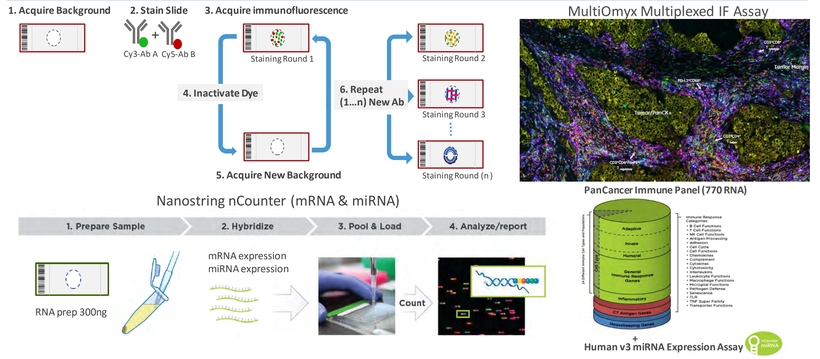






**Figure 1. Assay Workflow.** For MultiOmyx IF study, slides were prepared and stained using MultiOmyx multiplexing IF staining protocol. For each round of staining, conjugated fluorescent antibodies were applied to the slide, followed by imaging acquisition of stained slides. The dye was erased, enabling a second round of staining with another pair of fluorescent antibodies. For Nanostring nCounter assay, the RNA was extracted from the adjacent 10µm section and then proceeded with hybridization, purification and immobilization and count based on manufacturer's protocol.

## Conclusion

In this study, MultiOmyx and NanoString technologies, both platforms offered by Neogenomics Laboratories were utilized to measure miRNA, mRNA and protein expression in lung, head and neck, breast, and melanoma samples.

- Direct correlation was observed between NanoString nCounter values (mRNA) and percentage of positive cells measured by MultiOmyx IF multiplexed assay (protein) for 8 out of the 14 immuno-markers (CD3, CD4, CD8, S100, OX40, CD16, FOXP3 and GRANZYME B).
- No correlation was observed between mRNA and protein expression for the modulated markers (PD1, PD-L1, iCOS, HLA_DR). The differences observed may be due to the differences in cell population between the 4µm MultiOmyx section and the 10µm NanoString section, post-transcriptional regulation, and sample heterogeneity.
- Cluster analysis of the mRNA nCounter results revealed two main clusters. Low expression cluster, expressing OX40, OX40-L, FOXP3, PD1, and PD-L1. High expression cluster expressing CD4, CD8, ICOS, and GRANZYME B.
- MultiOmyx multiplexed IF assay delivered both quantitative cell classification and qualitative visualization of the tumor micro environment. The IF color overlaid images shown in Figure 3A-D, provide examples of unambiguous classification of OX40 on $T_{reg}$ cells (CD3+CD4+FOXP3+) and ICOS on $T_{helper}$ cells (CD3+CD4+).
- No statistically significant correlation was observed for miRNAs reported in literature as negative regulator of PD-L1, PD1, FOXP3 and iCOS. Targeted cell enrichment using laser capture microdissection (LCM) may be needed to further explore miRNAs regulation of immune checkpoint markers.

This study demonstrated MultiOmyx and NanoString technologies are complementary and enabled integrative analysis of protein, mRNA, and miRNA expression.



## mRNA Expression Profiling using Nanostring nCounter Assay

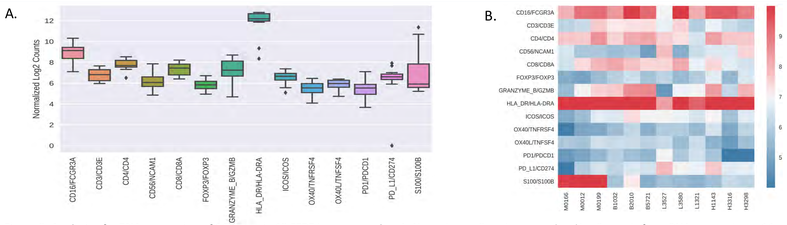



**Figure 2. Analysis of mRNA expression of 14 genes in 12 FFPE tumor samples.** A. Nanostring nCounter normalized Log2 counts for mRNA expression in 14 gene transcripts. B. Heat map of mRNA expression normalized log2 counts in 12 FFPE tumor samples.

## Immuno-Profiling using MultiOmyx Multiplexed Immunofluorescence Assay

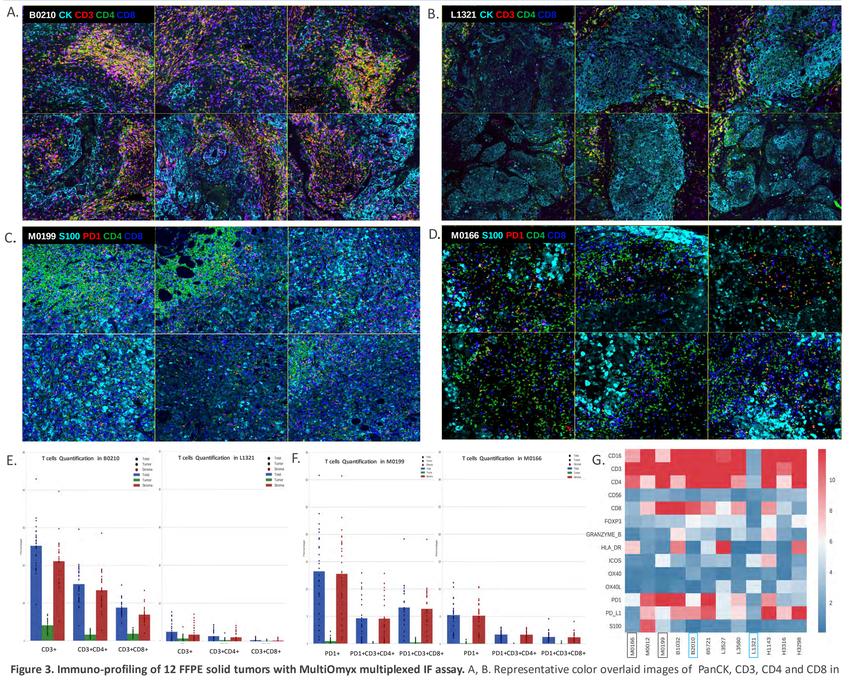
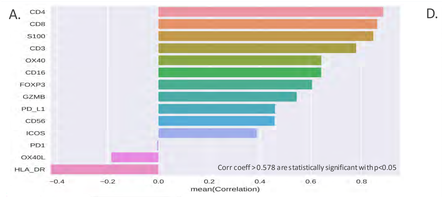
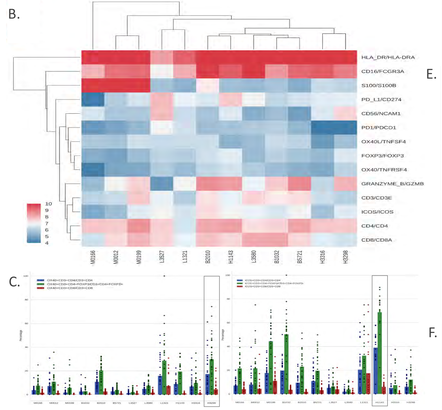



**Figure 3. Immuno-profiling of 12 FFPE solid tumors with MultiOmyx multiplexed IF assay.** A, B. Representative color overlaid images of PanCK, CD3, CD4 and CD8 in breast tumor B0210 and Lung tumor L1321. C, D. Representative color overlaid images of S100, PD1, CD4 and CD8 in two melanoma samples: M0199 and M0166. E. F. Quantification of immune markers utilizing MultiOmyx proprietary cell classification algorithm. The percentage of the positive T cell phenotypes are calculated and plotted. The bar represents the slide level result and each dot represents the data from one specific region of interest. G. Heat map of MultiOmyx immune cell classification results. The results are given as percentage of positive cells to all QC passed cells.

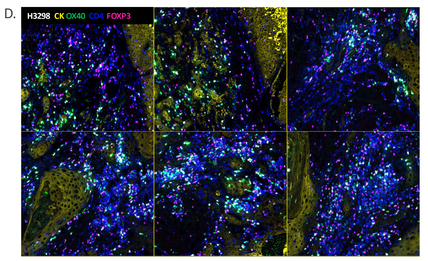
## Integrated mRNA and Protein Expression Analysis using Nanostring and MultiOmyx Technologies

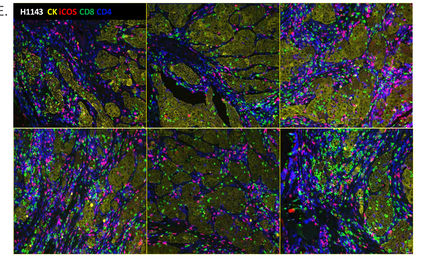








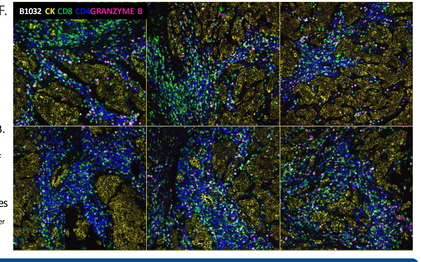
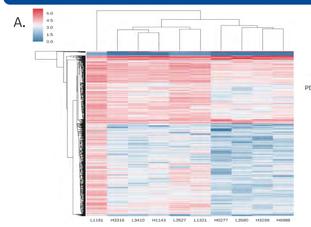




**Figure 5. Correlation between mRNA and protein.** A. Correlation coefficient for mRNA nCounter counts and percentage of positive cells measured by MultiOmyx showed correlation for 8/14 markers (CD4, CD8, S100, CD3, OX40, CD16, FOXP3, GRANZYME B). B. Cluster map of mRNA normalized log2 counts in 12 FFPE tumor samples revealed 2 main clusters. C. Quantification of immune markers in MultiOmyx assay showed percentage of OX40 and iCOS expression in different subtypes of T cells. OX40 expression is highest in $T_{reg}$ relative to $T_{helper}$ and $T_{cytotoxic}$. D. Color overlaid images of PanCK, OX40, CD4 and FOXP3 in H&N tumor H3298 showed OX40 expression in $T_{reg}$ cells. E. Color overlaid images of PanCK, iCOS, CD4 and CD8 in H&N tumor H1143 showed iCOS expression in both $T_{helper}$ and $T_{reg}$. F. Color overlaid images of PanCK, GRANZYME B, CD4 and CD8 in breast tumor B1032 showed GRANZYME B expression primarily in $T_{cytotoxic}$ cells.

## Correlation Assessment of miRNA and mRNA Expression Profiling



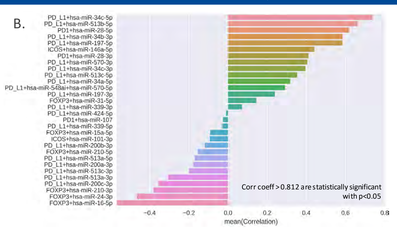




**Figure 6. Assessment of miRNA.** A. Cluster map of miRNA normalized log2 counts in FFPE tumor samples.
B. Correlation coefficient between miRNA and mRNA nCounter values. None of the miRNAs reported in the literature met the statistically significant threshold of > 0.812.