

Characterizing CD39 and CD73 cell subtypes in the tumor microenvironment using MultiOmyx™ immunofluorescence assay

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CD39 and CD73 are membrane bound enzymes that function together to convert extracellular adenosine 5'-triphosphate (ATP)/adenosine diphosphate (ADP) into adenosine. CD39 first catalyzes ATP/ADP into adenosine monophosphate (AMP), which is then converted by CD73 into adenosine. Accumulation of extracellular adenosine creates an immunosuppressive tumor environment and facilitates tumor growth and metastasis. Conversely, the presence of ATP promotes a pro-inflammatory, tumor-suppressive environment. Suppression of CD73/CD39 activity and reduction of extracellular adenosine has been shown to support an antitumor immune response. Therefore, the targeting of CD73 and CD39, both individually as well as in combination with immune checkpoint inhibitors for biomarkers such as PD-1 and CTLA-4, is an emerging strategy for cancer therapeutics.

CD73/CD39 has been described in multiple cell types, including tumor cells, fibroblasts, endothelial cells, tumor infiltrating lymphocytes (TILs), myeloid cells, and natural killer (NK) cells. While known to be expressed on multiple cell types, the spatial characterization of CD39 and CD73 in the tumor microenvironment (TME) is still poorly understood. Characterizing the diversity of CD39 and CD73 expressing populations in the tumor environment will help improve development of targeted therapies for cancer treatment.

In this study, we used the multiplex immunofluorescence (mIF) platform MultiOmyx to characterize the distinct populations of cells expressing CD39 and/or CD73 in the TME of a pan-cancer cohort, including 6 tissue microarrays (TMAs) from colon, prostate, and lung cancer indications. MultiOmyx is a proprietary mIF platform for the visualization and characterization of up to 60 protein biomarkers in a single FFPE section. The panel used in this study includes CD3, CD4, CD8, CD11b, CD31, CD39, CD68, CD73, CTLA-4, FOXP3, Granzyme B, HLA-DR, LAG3, NKp46, PD-1, TIM3, SMA, and tumor marker PanCK. This panel therefore enables the detection and characterization of CD39+ or CD73+ cell expression in immune, stroma, and tumor populations. Using proprietary deep-learning based image analysis, we are able to quantify the occurrence and densities of the different CD39 and CD73 positive cells and characterize each in different immune and other TME subtypes. Understanding the variety and phenotype of CD39 and CD73 expressing cells in the TME is crucial to define the populations being targeted by therapies for cancer treatment.

MultiOmyx Assay Workflow and Biomarker Panel

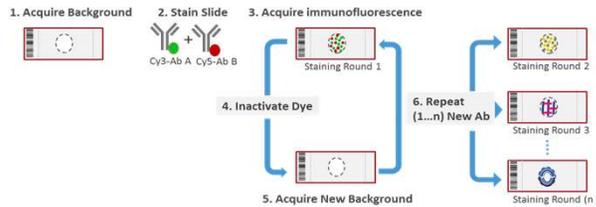


Figure 1. MultiOmyx Assay Workflow. Each sample was analyzed by MultiOmyx IF assay. For MultiOmyx IF study, slides were prepared and stained using MultiOmyx multiplexing IF staining protocol. For each round of staining, conjugated fluorescent antibodies were applied to the slide, followed by imaging acquisition of stained slides. The dye was erased, enabling a second round of staining with another pair of fluorescent antibodies.

Panel Biomarkers	MultiOmyx CD39 and CD73 Biomarkers and Representative Phenotypes			
	Co-expression	Phenotypes	Co-expression	Phenotypes
CD3	CD3+ CD4+ CD39+	CD39+ T helper cells	CD68+ CD39+	CD39+ Macrophage
CD4	CD3+ CD4+ FOXP3+ CD39+	CD39+ T regulatory cells	CD11b+ HLA-DR-CD39+	CD39+ MDSC
CD11b	CD3+ CD4+ CTLA4+ CD39+	CD34+ CTLA4+ T helper cells	NKp46+ CD39+	CD39+ NK cells
CD31	CD3+ CD4+ PD-1+ CD39+	CD34+ PD-1+ T helper cells	SMA+ CD39+	CD39+ Fibroblasts
CD39	CD3+ CD4+ TIM3+ CD39+	CD34+ TIM3+ T helper cells	CD31+ CD39+	CD39+ Blood Vessels
CD68	CD3+ CD8+ CD39+	CD39+ T cytotoxic cells	CD68+ CD73+	CD73+ Macrophage
CD73	CD3+ CD8+ Granzyme B+ CD39+	CD39+ GzB+ T cytotoxic cells	CD11b+ HLA-DR-CD73+	CD73+ MDSC
CTLA4	CD3+ CD8+ CTLA4+ CD39+	CD39+ CTLA4+ T cytotoxic cells	NKp46+ CD73+	CD73+ NK cells
FOXP3	CD3+ CD8+ PD1+ CD39+	CD39+ PD1+ T cytotoxic cells	SMA+ CD73+	CD73+ Fibroblasts
Granzyme B	CD3+ CD8+ TIM3+ CD39+	CD39+ TIM3+ T cytotoxic cells	CD31+ CD73+	CD73+ Blood Vessels
HLA-DR	CD3+ CD8+ LAG3+ CD39+	CD39+ LAG3+ T cytotoxic cells	PanCK+ CD73+	CD73+ Tumor
LAG3	CD3+ CD8+ LAG3+ CD39+	CD39+ LAG3+ T cytotoxic cells	PanCK+ CD73+	CD73+ Tumor
NKp46				
PD1				
PanCK				
SMA				
TIM3				

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Comprehensive Characterization of CD39 and CD73 Using MultiOmyx Assay

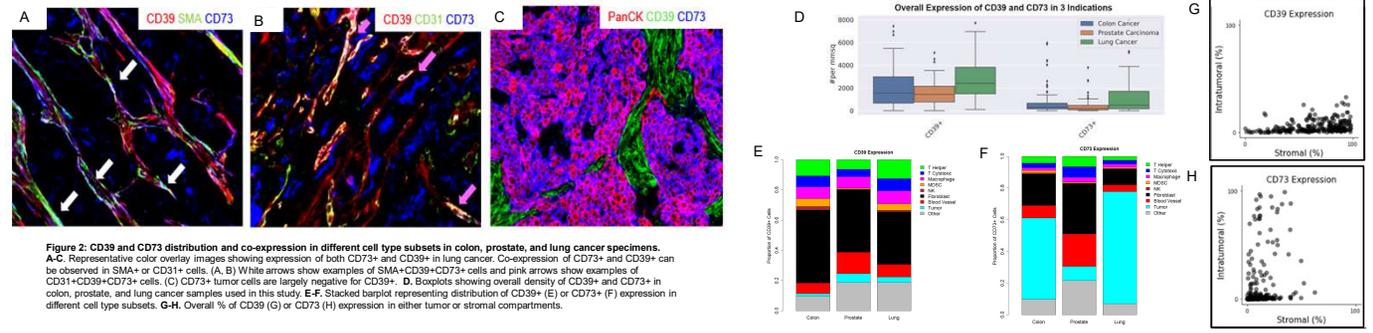


Figure 2. CD39 and CD73 distribution and co-expression in different cell type subsets in colon, prostate, and lung cancer specimens. A-C. Representative color overlay images showing expression of both CD73+ and CD39+ in lung cancer. Co-expression of CD73+ and CD39+ can be observed in SMA+ or CD31+ cells. (A, B) White arrows show examples of SMA+CD39+CD73+ cells and pink arrows show examples of CD31+CD39+CD73+ cells. (C) CD73+ tumor cells are largely negative for CD39+. D. Boxplots showing overall density of CD39+ and CD73+ in colon, prostate, and lung cancer samples used in this study. E-F. Stacked barplot representing distribution of CD39+ (E) or CD73+ (F) expression in different cell type subsets. G-H. Overall % of CD39 (G) or CD73 (H) expression in either tumor or stromal compartments.

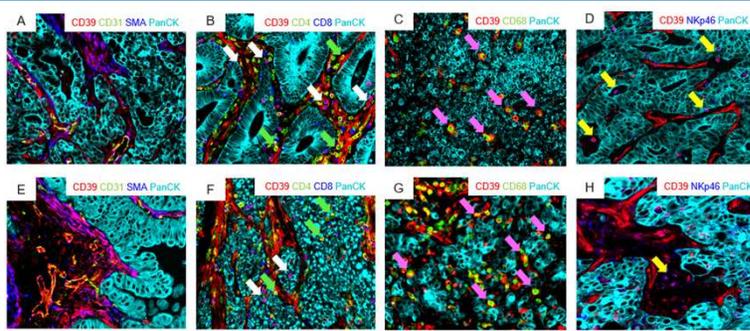


Figure 3. Characterization of CD39 expression in different cell type subsets in colon, prostate, and lung cancer specimens. A-H. Representative color overlay images showing expression of CD39 in colon (A-D) and lung (E-F) cancer specimens. (A, E) Co-expression of CD39+ with CD31+ or SMA+ cells appears as yellow (CD39+ CD31+ or magenta (CD39+ SMA+), respectively. (B, F) Green arrows indicate examples of CD39+ T helper cells (CD39+ CD31+) and white arrows indicate examples of CD39+ T cytotoxic cells (CD39+ CD39+). (C, G) Pink arrows show examples of CD39+ macrophages (CD39+ CD68+). (D, H) Yellow arrows show examples of CD39+ NK cells (CD39+ NKp46+). I. Boxplots comparing the densities of CD39+ expressing subsets in colon, prostate, and lung cancer samples used in this study.

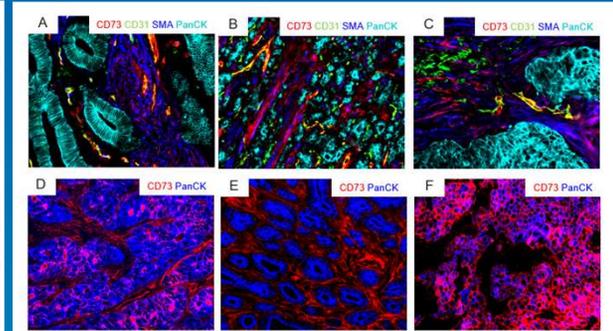
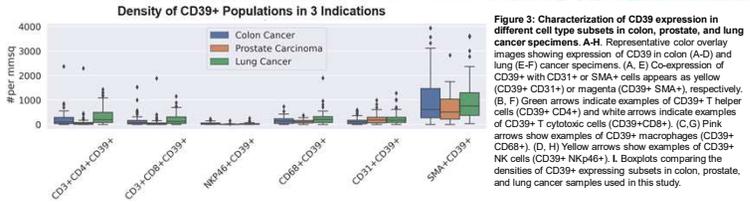


Figure 4. Characterization of CD73 expression in different cell type subsets in colon, prostate, and lung cancer specimens. A-F. Representative color overlay images showing expression of CD73 in colon (A-D), prostate (B, E), and lung (C, F), cancer specimens. (A-B) Co-expression of CD73+ with CD31+ or SMA+ cells appears as yellow (CD73+ CD31+) or magenta (CD73+ SMA+), respectively. (D, F) CD73+ tumor (CD73+ PanCK+) appears in magenta. (E) Prostate carcinoma was predominantly negative for CD73+ tumor expression. G. Boxplots comparing the densities of CD73+ expressing subsets in prostate carcinoma, NSCLC, and colon carcinoma samples used in this study.

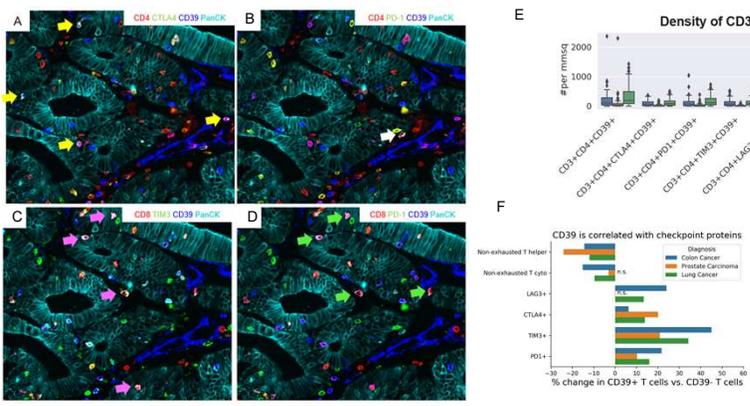
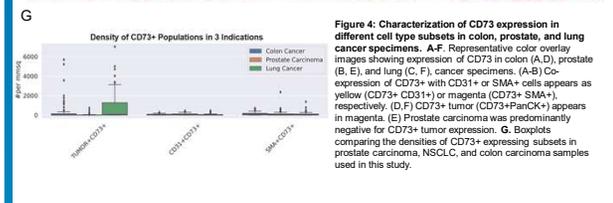


Figure 5. Characterization of CD39+ expression within immune checkpoint T cell subsets in colon, prostate, and lung cancer specimens. A-D. Representative color overlay images showing co-expression of CD39+ in colon carcinoma immune checkpoint T cell populations. (A) Yellow arrows show examples of CTLA4+ T helper cells expressing CD39 (CD39+ CTLA4+). (B) White arrow shows examples of PD-1+ T helper cell (CD39+ CD4+ PD-1+). (C) Pink arrows show examples of TIM3+ T cytotoxic cells expressing CD39 (CD39+ CD8+ TIM3+). (D) Green arrows show examples of PD-1+ T cytotoxic cells expressing CD39 (CD39+ CD8+ PD-1+). E. Boxplots comparing the densities of CD39+ expressing immune checkpoint T cell subsets in colon, prostate, and lung cancer samples used in this study. F. Percent change in expression of immune checkpoint proteins in CD39+ tumor infiltrating T cells compared to their CD39- counterparts. All results are significant except those marked n.s., based on bootstrapped 95% confidence intervals.

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- CD39 is more abundant than CD73 in colon, prostate, and lung cancer samples analyzed.
 - CD39 is primarily expressed in fibroblasts, blood vessels, and immune cells in colon, prostate, and lung cancer samples analyzed.
 - CD73 is primarily expressed in tumor, fibroblasts, and blood vessels in colon, prostate, and lung cancer samples analyzed.
 - Prostate samples show lower abundance of CD73+ tumor relative to lung or colon cancer samples.
 - CD39+ is co-expressed with immune checkpoint positive T cell populations in colon, prostate, and lung cancer samples.

