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Spatial interrogation of tertiary lymphoid structures (TLS) in colorectal carcinoma (CRC) tumor microenvironment (TME) using the MultiOmyx™ assay

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Immune checkpoint blockade (ICB) therapy has revolutionized the landscape of cancer therapy in multiple tumor types since Ipilimumab, the first ICB agent, was approved for the treatment of metastatic melanoma is 2011. Current predictive biomarkers for therapy response include PD-L1 expression, tumor mutational burden (TMB) and microsatellite instability (MSI) status. However, responses to ICB vary widely and these predictive markers have demonstrated imperfect predictive power to ICB therapy. For instance, half of microsatellite instability-high (MSI-H) colorectal carcinoma (CRC) patients do not respond to ICB immunotherapy. There is still a crucial need to identify and develop biomarkers predictive of outcome to ICB therapy. Tertiary lymphoid structures (TLS) have been observed in a variety of solid tumors in humans, and their presence is a favorable prognostic indicator for survival in a variety of solid cancers including CRC. Further, it was recently shown that the presence of mature TLS was associated with improved response rate to ICB and overall survival in patients with advanced tumors in a large retrospective study.

In this study, we used a 17-plex MultiOmyx panel to detect TLS, explore TLS maturation stage and characterize the tumor microenvironment (TME) in 40 CRC patients with known MSI status. The 17-plex includes CD3, CD4, CD8, CD45RO, FOXP3, CD20, CD56, CD68, CTLA-4, PD1, PD-L1, CXCL13, PNAd, CD21, CD23, DC-LAMP and PanCK. Using this panel, we successfully detected different stages of TLS in these CRC samples. Using proprietary deep-learning-based image analysis (NeoLYTX), we quantified TLS's in the CRC samples and classified them by maturation stage based on biomarker expression and spatial organization of immune cells. While there was no difference in the number of early TLS (E-TLS; characterized by CD3, CD20 and pNAd) between MSI-H and microsatellite-stable (MSS) CRC samples, more primary follicle-like TLS (P-TLS; defined by CD3, CD20 and CD21) and secondary follicle-like TLS (S-TLS; defined by CD3, CD20 and CD23) were observed in the MSI-H group than the MSS group. In particular, the abundance of mature S-TLS was significantly higher in MSI-H CRC samples.

This 17-plex MultiOmyx assay provides a powerful tool to characterize the cellular composition and spatial organization of the tumor microenvironment. The panel enables quantification of TLS, PD-L1 expression and abundance of tumor infiltrating lymphocytes (TIL) from one single FFPE slide. The rich datasets generated by the MultiOmyx assay can provide greater understanding of the immune contexture within the TME and deeper insights into the correlations between biomarkers. These findings may have broad application and help identify biomarker signatures with improved predictive performance to immune checkpoint inhibition efficacy in solid tumors.

MultiOmyx Assay Workflow and Biomarker Panel

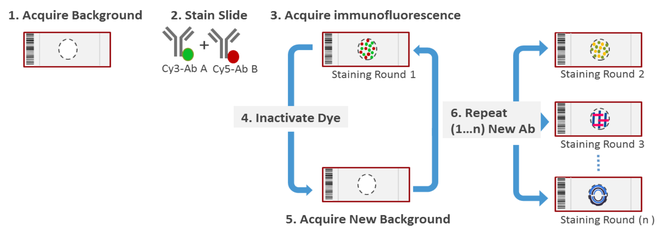
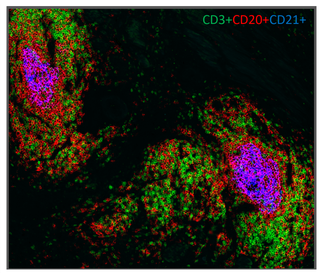


Figure 1. MultiOmyx Assay Workflow. Each sample was analyzed by MultiOmyx IF assay. For MultiOmyx IF study, slides were prepared and stained using MultiOmyx multiplexing IF staining protocol. For each round of staining, conjugated fluorescent antibodies were applied to the slide, followed by imaging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

17-plex Panel Biomarkers and Phenotypes

Panel Biomarkers	
CD3	CXCL13
CD4	PNAd
CD8	CD21
CD45RO	CD23
FoxP3	DC-LAMP
CD20	
CD68	
CD56	
CTLA-4	
PD-1	
PD-L1	
PanCK	

Co-expression	Phenotypes
CD3+CD4+	T helper
CD3+CD4+FoxP3+	T regulatory
CD3+CD4+CD45RO+	Memory T helper
CD3+CD4+PD1+	Immune modulation
CD3+CD8+	T cytotoxic
CD3+CD8+CD45RO+	Memory T cytotoxic
CD3+CD8+PD1+	Immune modulation
CD68+PDL1+	Macrophage PD-L1
PanCK+PDL1+	Tumor cell PD-L1
CD3+CD20+PNAd+	Early TLS
CD3+CD20+CD21+	Primary Follicle-like TLS
CD3+CD20+CD21+CD23+	Secondary Follicle-like TLS



Comprehensive Characterization of TLS using 17-plex MultiOmyx Assay

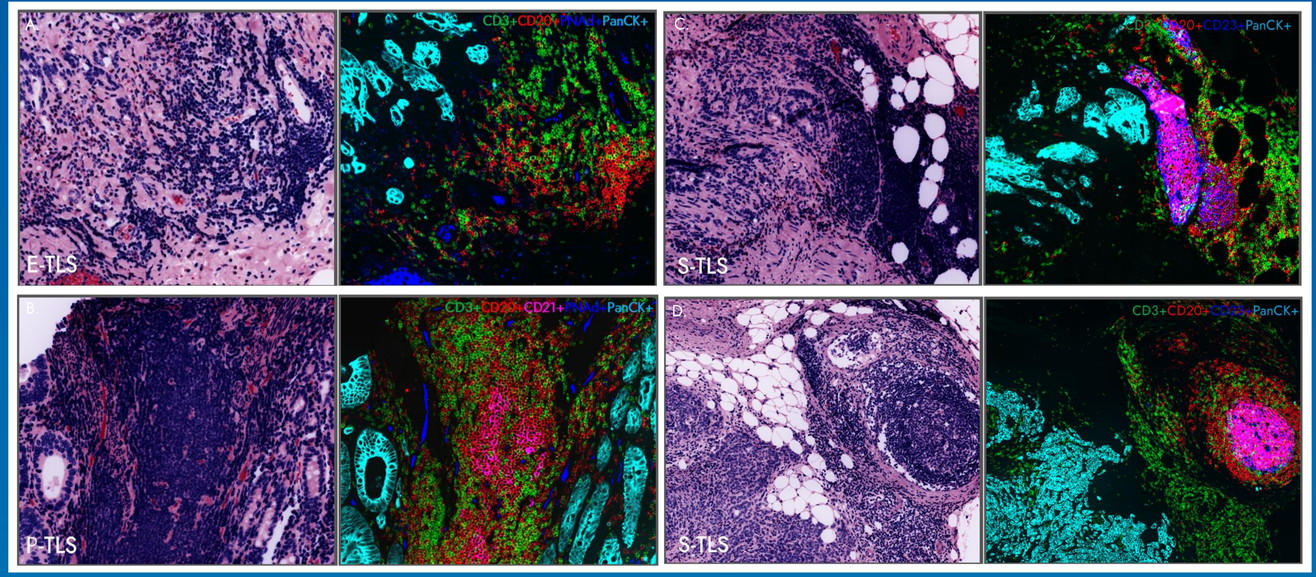
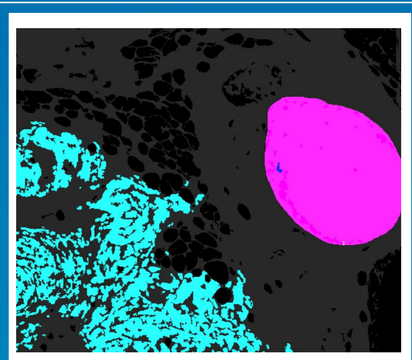


Figure 2. Characterization of different stages of TLS in CRC samples using MultiOmyx assay. **A.** Example of E-TLS. The example is positive for CD3, CD20 and PNAd. **B.** Example of P-TLS. Expression of CD21 is observed. **C&D.** Examples of S-TLS. The S-TLS are positive for CD23, indicating GC formation.

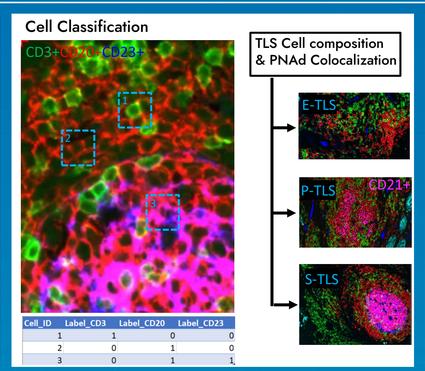
Key Study Highlights

- 17-plex MultiOmyx panel allows detection of TLS and comprehensive analysis of TLS with different maturation stages.
- The panel enables analysis of TLS, PD-L1 expression and abundance of TIL from one single FFPE slide.
- A powerful tool to characterize the cellular composition and spatial organization of the tumor microenvironment.

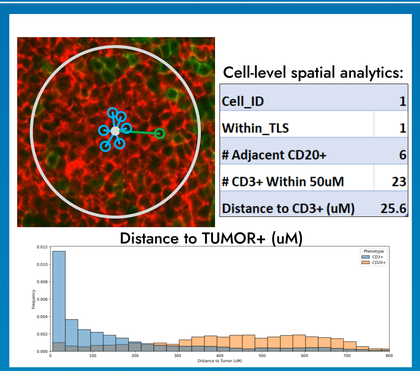
Quantification of TLS Using Proprietary Deep-learning Based Analytics Pipeline



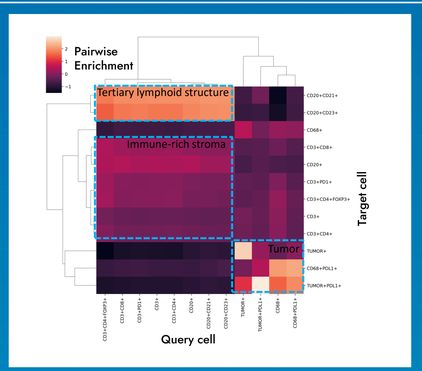
3A. Segmentation: Tissue (grey), Tumor (cyan), TLS (magenta), PNAd (blue), as well as nuclear segmentation using DAPI (not shown)



3B. Classification: Cells are classified by biomarker, and TLS's are classified into stages according to cell composition and PNAd colocalization. Magenta corresponds to CD20+CD23+ cells except where indicated to be CD21+



3C. Spatial analytics: Cells are categorized according to tissue region and proximity to other cell types. Histogram shows the distance to Tumor for CD3+ and CD20+ cells from a representative sample (Fig. 2D)



3D. Pairwise Enrichment: Cell phenotypes are clustered based on proximity (within 50uM) between query and target cell phenotypes for a representative MSI-H CRC sample (Fig. 2D).

Results and Summary

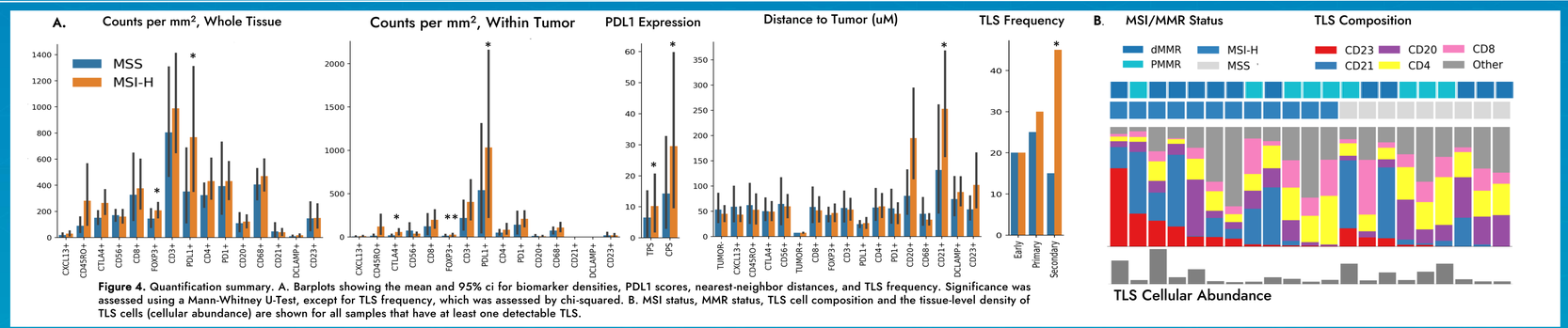


Figure 4. Quantification summary. A. Barplots showing the mean and 95% ci for biomarker densities, PDL1 scores, nearest-neighbor distances, and TLS frequency. Significance was assessed using a Mann-Whitney U-Test, except for TLS frequency, which was assessed by chi-squared. B. MSI status, MMR status, TLS cell composition and the tissue-level density of TLS cells (cellular abundance) are shown for all samples that have at least one detectable TLS.

- Mature TLS are significantly higher in MSI-H CRC group, with CD21+ cells significantly farther from the tumor region.
- Higher PD-L1 expression in MSI-H CRC group.
- More CTLA4+ and FOXP3+ cells infiltrated into the tumor regions in MSI-H CRC group.

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