# SITC 2022 Abstract#87

## Deep phenotyping and spatial immune contexture characterization through a combined multiplex immunofluorescence (mIF) approach

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**Background**: Tertiary lymphoid structures (TLS) have been observed in a variety of solid tumors and growing evidence has shown that TLS can be promising prognostic indicators of positive outcomes for patients with solid tumors including colorectal cancer (CRC) [1]. Large-scale retrospective analysis shows patients with mature TLS in particular respond to PD-1/PD-L1 antibody treatment with improved objective response, progression-free and overall survival [2]. Since not all patients respond to immune checkpoint blockade (ICB) therapy, identifying patients with TLS can be clinically relevant as it enables selection of patients likely to respond. In this study, we demonstrate a tissue-based phenotyping workflow combining two complementary multiplex immunofluorescence (mIF) platforms to enable identification of TLS, characterization of TLS maturation stage and spatial phenotyping of tumor infiltrating lymphocytes (TIL) on whole slide scan.
**Methods**: In this study, CRC samples were first stained with an in-house developed PhenoImager™ mIF panel detecting CD20, CD21, CD23, CD3 and Cytokeratin. Whole slide scan was acquired using PhenoImager HT, followed by biomarker classification and TLS identification using custom analytics algorithm generated with Indica HALO platform.
An adjacent section of tumor sample was then stained and analyzed with a 17-plex MultiOmyx™ mIF panel. Using the MultiOmyx assay in combination with proprietary deep-learning-based image analysis (NeoLYTX), we further characterized the TLS maturation stage and interrogated the correlation of the TLS presence with subtypes of TIL expression in the CRC samples.
**Results**: PhenoImager 5-plex TLS panel combined with Halo custom analysis successfully identified TLS in the tumor microenvironment (TME) of CRC samples and led to the strategic selection of ROIs for further characterization by high-plex assay. The TLS detection was found to be concordant between both mIF platforms in the CRC samples. MultiOmyx 17-plex analysis was able to provide a detailed picture of TLS and enabled further classification of TLS into different maturation stages based on biomarker expression and spatial organization of immune cells in the CRC samples.
**Conclusion**: Combination of PhenoImager and MultiOmyx IF provides a complementary and powerful solution to study cellular composition within the TME. PhenoImager assay characterizes the immunophenotypes and visualizes the spatial distribution of TIL at single-cell resolution on whole slides. High dimensional analysis by MultiOmyx can provide greater understanding of the immune contexture within the TME and deeper insights into the correlations between biomarkers. This combined approach may have broad application and provides novel insights into the complex TME.

## Study Workflow and Biomarker Panel

Section 1: PhenoImager (PI) 5-plex


Section 2: MultiOmyx (MO) 17-plex

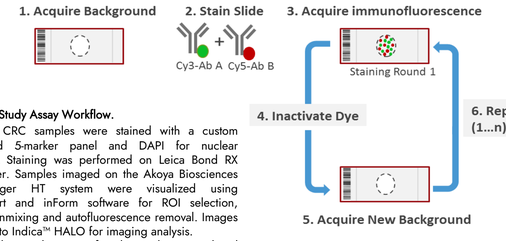
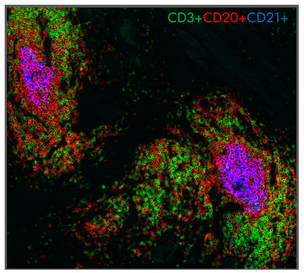



**Figure 1. Study Assay Workflow.**
For PI, CRC samples were stained with a custom developed 5-marker panel and DAPI for nuclear detection. Staining was performed on Leica Bond RX Autostainer. Samples imaged on the Akoya Biosciences PhenoImager HT system were visualized using Phenochart and inForm software for ROI selection, spectral unmixing and autofluorescence removal. Images imported to Indica™ HALO for imaging analysis.
For MO, the serial section of each sample was analyzed by MO 17-plex assay. The slides were prepared and stained using MO 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. The images were then analyzed using in-house developed NeoLYTX algorithms.




**PI 5-plex Panel Biomarkers and Phenotypes**

| Panel Biomarkers | |
|---|---|
| CD3 | CD21 |
| CD20 | CD23 |
| CK | |

| Co-expression | Phenotypes |
|---|---|
| CD3+CD20+CD21+ | Mature TLS |
| CD3+CD20+CD21+CD23+ | |

**MO 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/ Co-localization | 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 |



# Concordant Detection of TLS using 5-plex PI Assay and 17-plex MO Assay

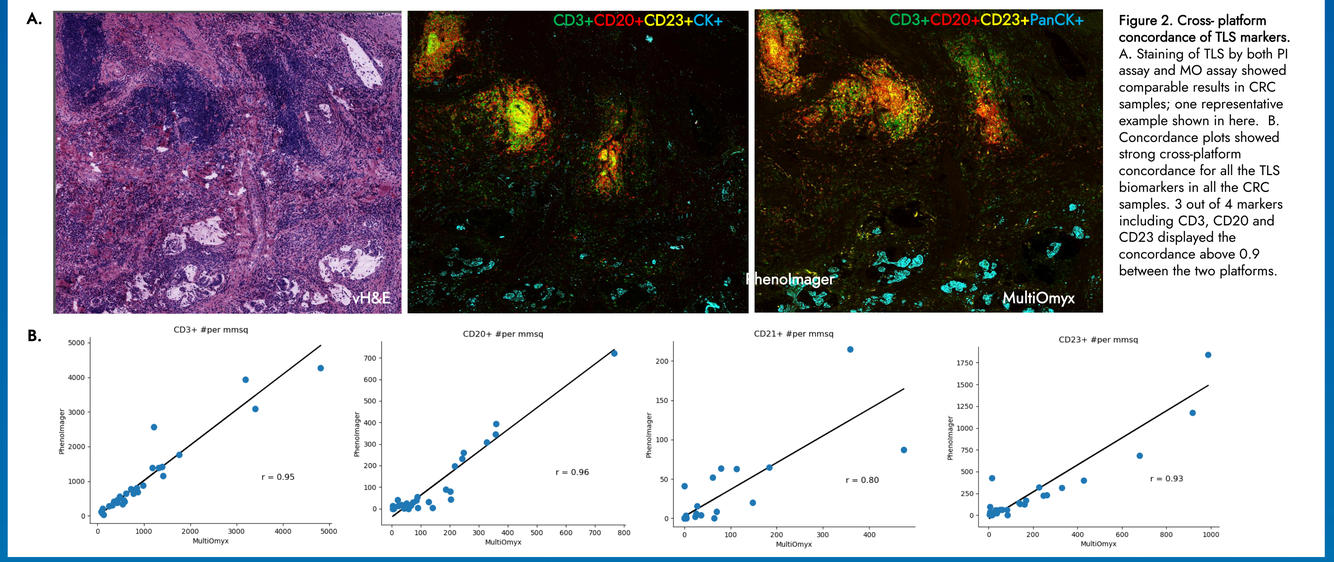



**Figure 2. Cross- platform concordance of TLS markers.** A. Staining of TLS by both PI assay and MO assay showed comparable results in CRC samples; one representative example shown in here. B. Concordance plots showed strong cross-platform concordance for all the TLS biomarkers in all the CRC samples. 3 out of 4 markers including CD3, CD20 and CD23 displayed the concordance above 0.9 between the two platforms.

## Key Study Highlights

- Highly concordant data was observed between the two mIF platforms used in this study.
- Combination of PhenoImager and MultiOmyx IF provides a complementary and powerful solution to study cellular composition within the TME.
- PhenoImager assay characterizes the immunophenotypes and visualizes the spatial distribution of TIL at single-cell resolution on whole slides.
- High dimensional analysis by MultiOmyx can provide greater understanding of the immune contexture within the TME and deeper insights into the correlations between biomarkers.
- This combined approach may have broad application and provides novel insights into the complex TME.

## Concordant Identification of TLS using Halo and NeoLYTX

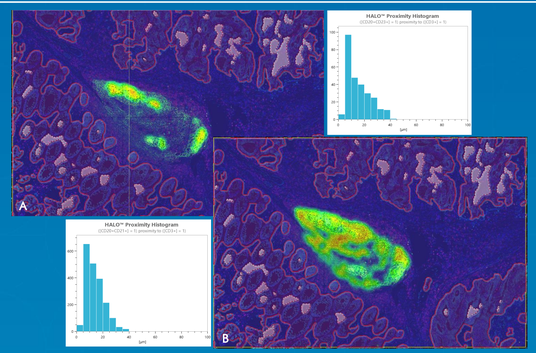
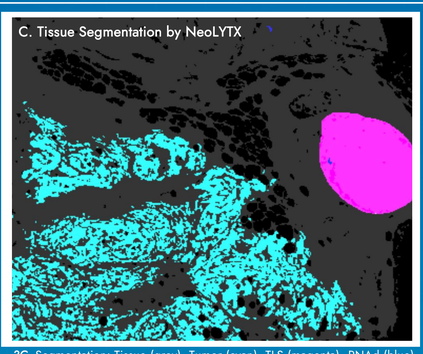
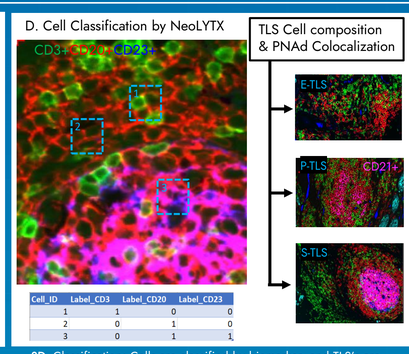


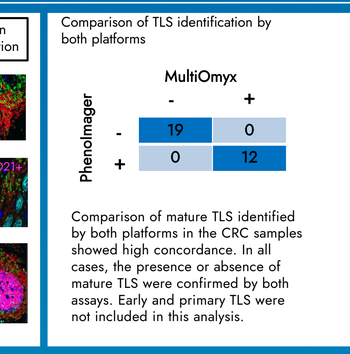

Figure 3. Halo Proximity histogram of A) CD23 containing and B) CD21 containing hotspots detecting tissue area containing panel biomarkers




3C. Segmentation: Tissue (grey), Tumor (cyan), TLS (magenta), PNAd (blue), as well as nuclear segmentation using DAPI (not shown). Putative TLS were identified using the DBSCAN clustering algorithm on CD20+ and CD3+ cells, and further phenotyped in 3D




3D. 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+

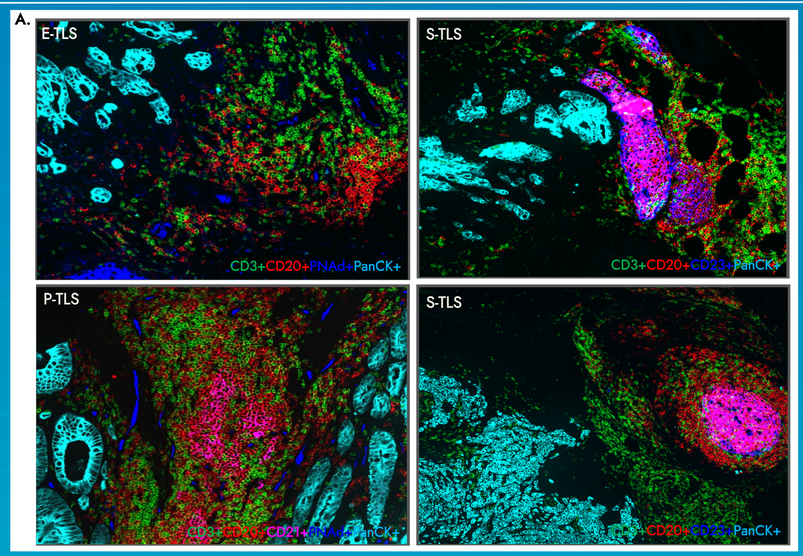


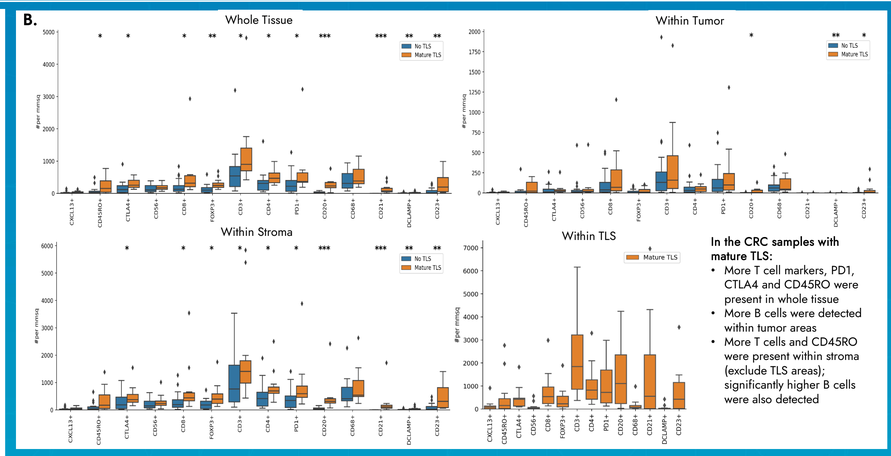

Comparison of TLS identification by both platforms

| PhenoImager \ MultiOmyx | - | + |
|---|---|---|
| - | 19 | 0 |
| + | 0 | 12 |

Comparison of mature TLS identified by both platforms in the CRC samples showed high concordance. In all cases, the presence or absence of mature TLS were confirmed by both assays. Early and primary TLS were not included in this analysis.

## Characterization of TLS within the TME using MO 17-plex




**In the CRC samples with mature TLS:**
- More T cell markers, PD1, CTLA4 and CD45RO were present in whole tissue
- More B cells were detected within tumor areas
- More T cells and CD45RO were present within stroma (exclude TLS areas); significantly higher B cells were also detected

**Figure 4. Characterization of TLS and IO markers within the TME by MO assay** A. Classification of TLS maturation stage by MO 17-plex assay. Examples of E-TLS, P-TLS and S-TLS. B. Barplots showing the mean and 95% ci for biomarker densities in whole tissue, within tumor, within stroma (excluding TLS area) and within TLS. Significance was assessed using a Mann-Whitney U-Test.

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