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SignalStar™ multiplex immunohistochemistry is a flexible spatial technology with fully validated protocols.

Background

Exploring spatial biology through multiplex immunohistochemistry (mIHC) empowers researchers to delve into the intricate functions, arrangements, and interactions of cells that shape the tumor microenvironment (TME) during disease progression or in response to therapy. SignalStar introduces an innovative mIHC assay employing oligo-conjugated antibodies and a matrix of fluorescent oligonucleotides, amplifying up to 8 targets within a single FFPE tissue.

Methods

Rigorous validation ensures optimal conjugation efficiency and ensures antibody sensitivity and specificity. The myeloid compartment of the TME was assessed using antibodies targeting CD11c, SIRPα, CD163, CD206, CD68, CD45, HLA-DRA, and Pan-Keratin. The simultaneous application of all antibodies is followed by amplifying the signal of four targets in the initial imaging round. Fluorescent signal was removed, and the signal of 4 additional antibodies were amplified in the second round of imaging. SignalStar staining was quantitatively compared to the established chromogenic gold standard to confirm reproducibility across modalities and between replicates.

Results

Our findings demonstrate the consistent performance of each antibody across all available fluorescent channels in multiple rounds of imaging regardless of manual or automated protocols, as exemplified by SIRPα and Pan-Keratin. Image alignment and quantification of target signal frequency and co-localization was performed using QuPath. Anticipated cell subsets expressing multiple biomarkers, such as quadruple CD206+CD68+CD163+SIRPα+ cells, were successfully identified. The SignalStar mIHC technology facilitates adaptable panel design without lengthy optimization while upholding precision and reproducibility in quantifiable phenotypic data for comprehensive immune cell interrogation within the TME.

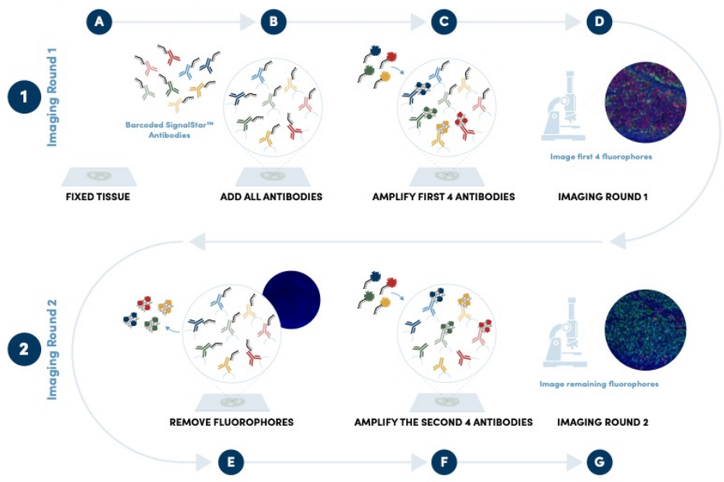
Conclusions

Spatial imaging is increasingly important to understand the different biological aspects of the TME, including the cells present and the organization and function of their biomarkers. This can be critical to understanding the differences between diseased and healthy tissue. Current technology is laborious and time consuming, particularly when it comes to higher throughput needs, increasing the time and costs of research.

SignalStar mIHC technology has established an assay that circumvents these issues, providing a sensitive, reproducible, and customizable assay for comprehensive biomarker spatial imaging of fixed tissues. Obtaining results is 70% faster than other mIHC approaches with no need for time consuming assay optimization, and results are obtained in two days from start to finish, speeding up translational workflows.

In addition to being a trusted leader of high quality and rigorously tested antibodies, CST provides comprehensive technical support and educational assistance available for the set-up and follow through of your imaging experiments to ensure their successful completion and streamlining your research process.

Figure 1. SignalStar™ mIHC Technology Overview



How SignalStar Works. The SignalStar assay allows for the simultaneous detection of up to 8 targets in formalin-fixed paraffin embedded (FFPE) tissue. Deparaffinized and rehydrated FFPE tissue sections undergo antigen retrieval (A), and all antibodies in your plex-size of choice (3-8 maximum unique oligo-conjugated antibodies) are added in one primary incubation step (B). A network of complementary oligos with fluorescent dyes (excitations: 488, 594, 647, 750) amplify the signal of up to 4 antibodies in the first round of imaging (C-D). If the plex-size is greater than 4, the first round of oligonucleotides and fluorophores are gently and specifically removed (E), and a second round of amplification is performed to visualize up to 4 additional oligo-conjugated antibodies (F). The two images are then aligned and fused computationally with either proprietary or open-source software to generate the up to 8-plex image (G).

Figure 2. SignalStar Oligo-Conjugated Antibodies undergo Extensive Validation and Optimization

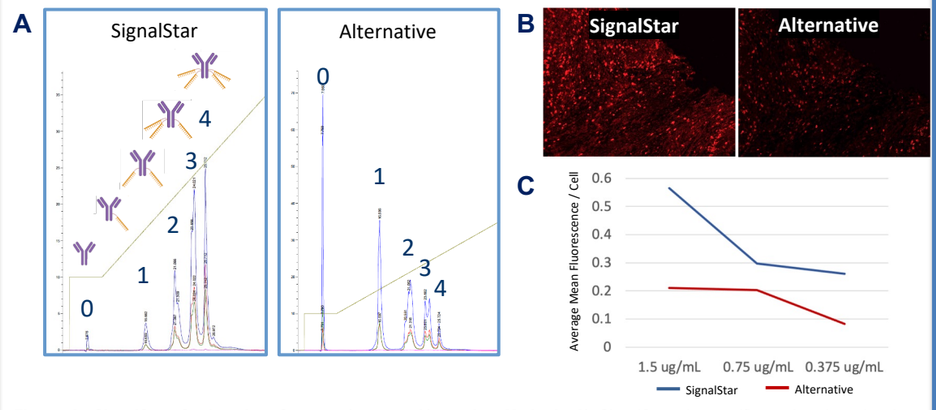


Figure 2. SignalStar Conjugation Compared to an Alternative Method. ***A)*** SignalStar site-specific conjugation chemistry consistently outperforms the alternative, resulting in a higher DOL and less unconjugated antibody as shown by analysis of partially purified samples. Advanced purification methods selectively isolate the optimal DOLs for each conjugate while ensuring complete removal of excess oligo and unconjugated antibody. ***B)*** SignalStar™ immunohistochemical analysis of paraffin-embedded human gastric adenocarcinoma using PD-1 (Intracellular Domain) (D4W2J) XP® Rabbit mAb (SignalStar™ Conjugate 0008) conjugated with CST methods (left, red) compared to PD-1 (Intracellular Domain) (D4W2J) XP® Rabbit mAb conjugated with alternative methods (right, red). All fluorophores have been assigned a pseudocolor, as indicated. Staining was performed on the BOND RX autostainer by Leica Biosystems. ***C)*** Mean fluorescence intensity of PD-1 positive cells was quantified for matching regions of interest (ROI) across indicated concentrations of conjugated antibodies.

Figure 3. Oligo-Conjugated Antibodies are Stable for 12 months

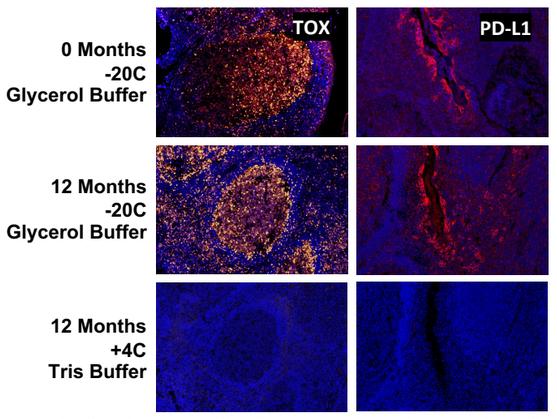


Figure 3. Stability of SignalStar Oligo-conjugated Antibodies. SignalStar immunohistochemical analysis of paraffin-embedded human tonsil using Tox/Tox2 (E6I3Q) & CO-0016-594 SignalStar™ Oligo-Antibody Pair #44575 (left, orange) or PD-L1 (E1L3N®) & CO-0005-647 SignalStar™ Oligo-Antibody Pair #52085 (right, red) stored at -20°C in storage buffer, containing PBS (pH 7.2), less than 0.1% sodium azide, 2 mM EDTA, 0.05% Triton X-100, 2 mg/mL BSA, and 50% glycerol, compared to storage at 4°C in AEX buffer, containing 20mM Tris and 10mM NaCl at pH 8.5, for indicated time points. All fluorophores have been assigned a pseudocolor, as indicated. Staining was performed on the BOND RX autostainer by Leica Biosystems.

Figure 4. Oligo-conjugated Antibodies are Validated for Flexibility

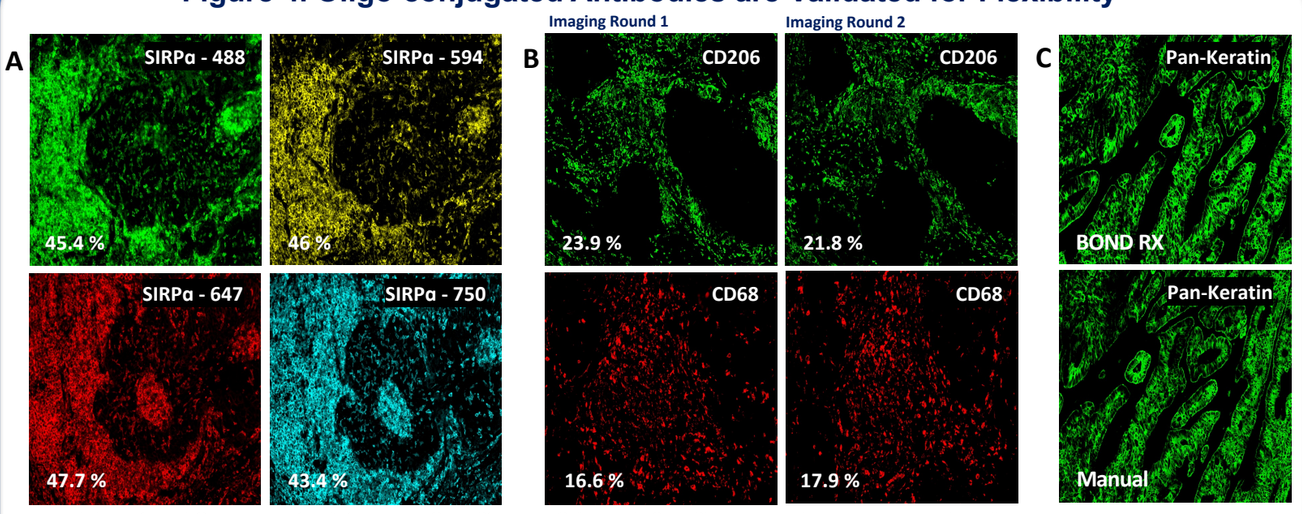


Figure 4. Validation of Oligo-conjugated Antibodies in SignalStar™ mIHC Assay. ***A)*** SignalStar immunohistochemical analysis of paraffin-embedded human squamous cell lung carcinoma using SIRPα (D6I3M) (SignalStar™ Conjugate 0034) in the 488 (green), 594 (yellow), 647 (red) & 750 (cyan) fluorescent channels. Percent positive cells in the representative ROI were quantified. ***B)*** SignalStar™ immunohistochemical analysis of paraffin-embedded human squamous cell lung carcinoma using CD206/MRC1 (E2L9N) & CO-0035-488 SignalStar™ Oligo-Antibody Pair #99626 (top, green) and CD68 (D4B9C) & CO-0007-647 SignalStar™ Oligo-Antibody Pair #83113 (bottom, red), showing consistent target expression across 2 imaging rounds. Percent positive cells in the representative ROI were quantified. All fluorophores have been assigned a pseudocolor, as indicated. Staining was performed on the BOND RX autostainer by Leica Biosystems. ***C)*** SignalStar immunohistochemical analysis of paraffin-embedded human colon carcinoma using Pan-Keratin (C11) & CO-0003-488 SignalStar™ Oligo-Antibody Pair #63566 (green). Target expression is consistent when the assay is performed on the BOND RX or performed manually.

Figure 5. Reproducible Results between 8-plex Replicates, Single-plex Stains, and Chromogenic DAB

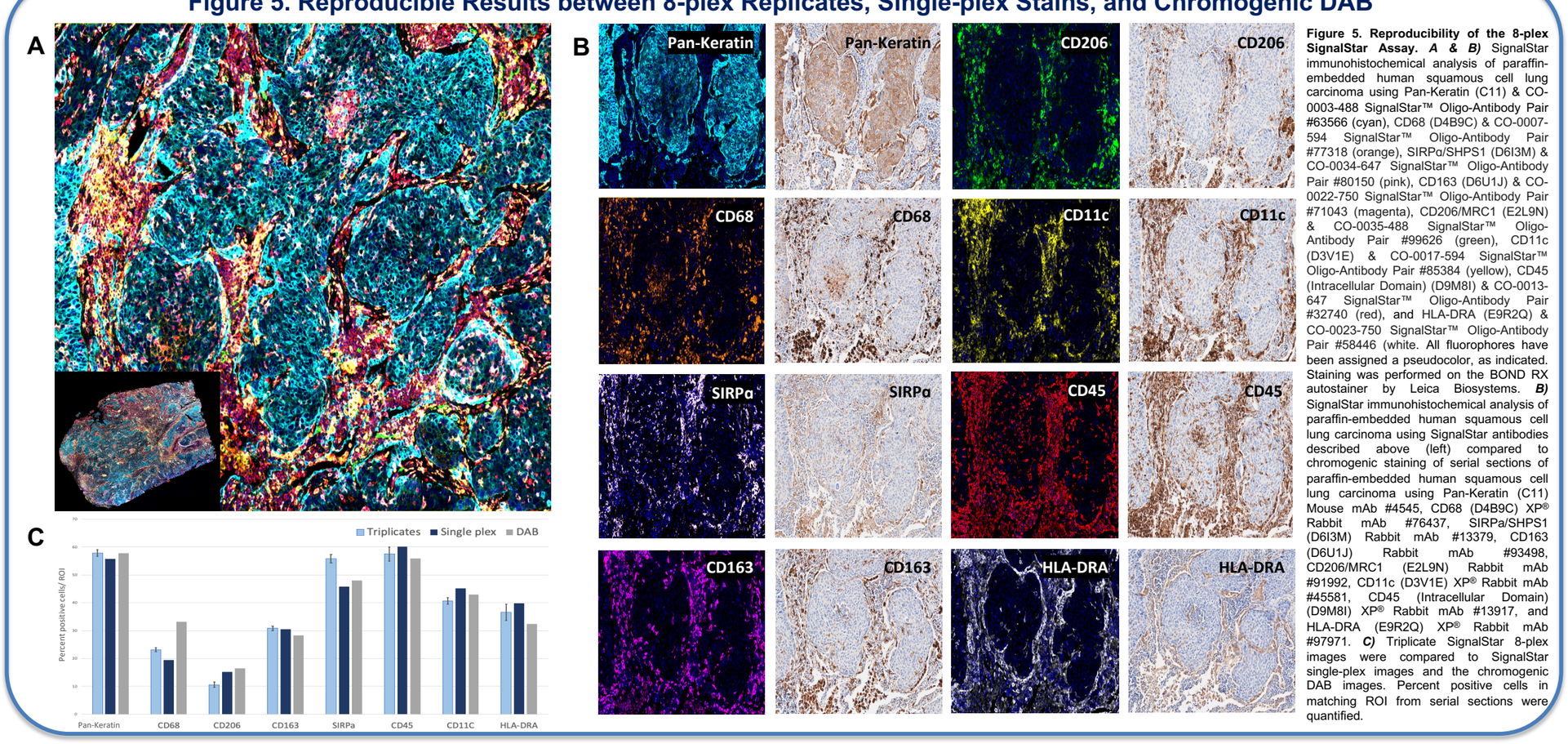


Figure 5. Reproducibility of the 8-plex SignalStar Assay. ***A & B)*** SignalStar immunohistochemical analysis of paraffin-embedded human squamous cell lung carcinoma using Pan-Keratin (C11) & CO-0003-488 SignalStar™ Oligo-Antibody Pair #63566 (cyan), CD68 (D4B9C) & CO-0007-594 SignalStar™ Oligo-Antibody Pair #77318 (orange), SIRPα/SHPS1 (D6I3M) & CO-0034-647 SignalStar™ Oligo-Antibody Pair #80150 (pink), CD163 (D6U1J) & CO-0022-750 SignalStar™ Oligo-Antibody Pair #71043 (magenta), CD206/MRC1 (E2L9N) & CO-0035-488 SignalStar™ Oligo-Antibody Pair #99626 (green), CD11c (D3V1E) & CO-0017-594 SignalStar™ Oligo-Antibody Pair #85384 (yellow), CD45 (Intracellular Domain) (D9M8I) & CO-0013-647 SignalStar™ Oligo-Antibody Pair #32740 (red), and HLA-DRA (E9R2Q) & CO-0023-750 SignalStar™ Oligo-Antibody Pair #58446 (white. All fluorophores have been assigned a pseudocolor, as indicated. Staining was performed on the BOND RX autostainer by Leica Biosystems. ***B)*** SignalStar immunohistochemical analysis of paraffin-embedded human squamous cell lung carcinoma using SignalStar antibodies described above (left) compared to chromogenic staining of serial sections of paraffin-embedded human squamous cell lung carcinoma using Pan-Keratin (C11) Mouse mAb #4545, CD68 (D4B9C) XP® Rabbit mAb #76437, SIRPa/SHPS1 (D6I3M) Rabbit mAb #13379, CD163 (D6U1J) Rabbit mAb #93498, CD206/MRC1 (E2L9N) Rabbit mAb #91992, CD11c (D3V1E) XP® Rabbit mAb #45581, CD45 (Intracellular Domain) (D9M8I) XP® Rabbit mAb #13917, and HLA-DRA (E9R2Q) XP® Rabbit mAb #97971. ***C)*** Triplicate SignalStar 8-plex images were compared to SignalStar single-plex images and the chromogenic DAB images. Percent positive cells in matching ROI from serial sections were quantified.

Figure 6. SignalStar mIHC Assay Enables Quantitative Analysis

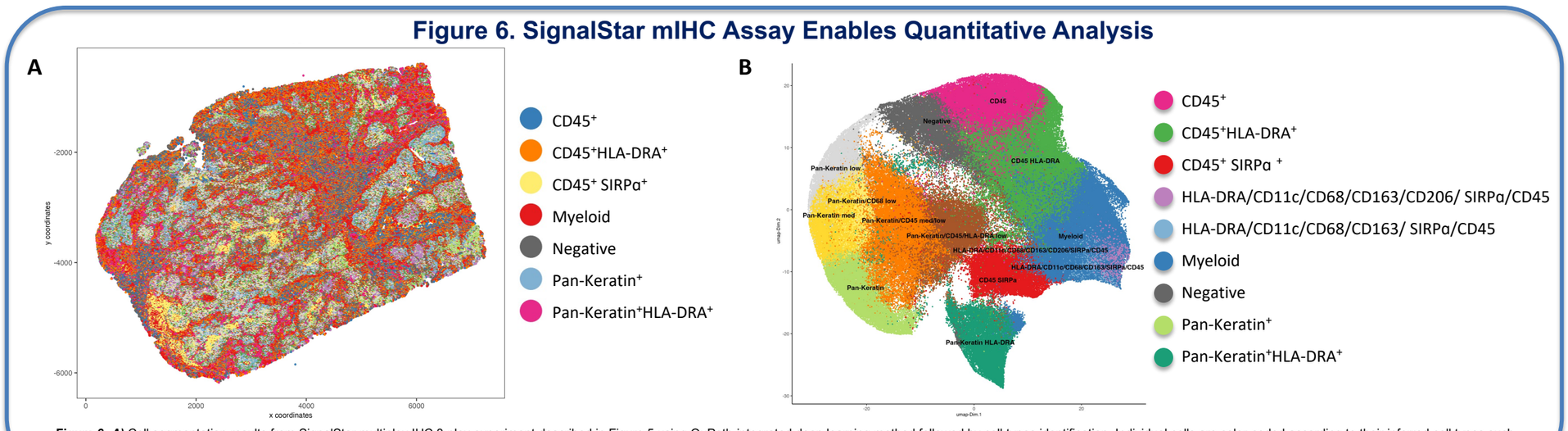


Figure 6. ***A)*** Cell segmentation results from SignalStar multiplex IHC 8-plex experiment described in Figure 5 using QuPath-integrated deep learning method followed by cell types identification. Individual cells are color-coded according to their inferred cell types such as myeloid, Pan-Keratin, and others. ***B)*** Cell type identification using Leiden clustering from the Giotto R package. Several cell types were identified including myeloid cells, epithelial cells (Pan-Keratin+) and HLA-DRA (MHC-II) expressing Pan-Keratin+ cells. Each cell is represented as a pair of UMAP coordinates.

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