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SignalStar™ multiplex immunohistochemistry is a novel technology capable of amplifying the signal of multiple biomarkers in the tumor microenvironment.

Background

There is a growing need to characterize multiple targets simultaneously in the tissue microenvironment (TME) in order to identify novel biomarkers that can be used to drive precision medicine. Multiplex immuno-histochemistry (mIHC) enables the imaging and quantification of multiple biomarkers at a single cell level while retaining spatial context. SignalStar mIHC by Cell Signaling Technology® is a fluorescent multiplexing approach that amplifies the signal of up to 8 protein biomarkers in FFPE tissues.

Methods

SignalStar oligo-conjugated antibodies were applied simultaneously to label markers of T cell phenotype and function (CD8α, PD-1, TIM-3, LAG3, Tox/Tox2, TCF1/TCF7 and Granzyme B) within the spatial context of the Pan-Keratin+ tumor. The signal of the first four targets was amplified and imaged, followed by removal of fluorescent signal, and the signal of 4 additional antibodies were amplifiedand imaged. In subsequent panels, PD-L1 and LAG3 were substituted into the 8-plex mIHC panel without additional protocol optimization required. The SignalStar assay underwent rigorous validation on tissue microarrays and whole tissues with diverse target expression levels to ensure assay reproducibility and dynamic range. SignalStar staining was compared to the chromogenic gold standard, tyramide signaling amplification (TSA), indirect fluorescent, and direct fluorescent labeling, and quantified with respect to signal intensity and frequency.

Results

Our data demonstrates consistent staining with SignalStar mIHC compared to the chromogenic DAB in serial sections across replicates. SignalStar amplification enabled detection of targets in tissues with high, medium, and/or low levels of target expression, and produced signal intensity comparable to TSA while substantially greater than direct or indirect immunofluorescence.

Conclusions

Multiplex IHC is ideal for maximizing the data acquired from limited FFPE tissue, and can provide a detailed map of the spatial distribution and expression patterns of multiple targets. Current spatial imaging techniques limit forward progress due to the time-consuming methods needed for assay design and antibody panel development, optimization, validation and analysis.

Our data demonstrates SignalStar mIHC can eliminate these hurdles by providing customizable and highly validated antibody panels that work right out of the box. Here, the ability to rapidly characterize biomarkers of T-cell exhaustion with ease and flexibility is demonstrated, enabling powerful phenotypic characterization with respect to signal intensity and expression patterns of multiple biomarkers. As new targets are identified and research needs evolve, researchers can switch out targets and quickly redesign panels without the need for additional panel or protocol optimization, as SignalStar antibodies and fluorophores are interchangeable.

Importantly, SignalStar provides amplification of signal for detection of targets with low expression. Our data indicates SignalStar can enable detection of low abundance proteins that may be beyond the detection limit for direct and indirect immunofluorescence.

Figure 1. SignalStar mIHC technology overview

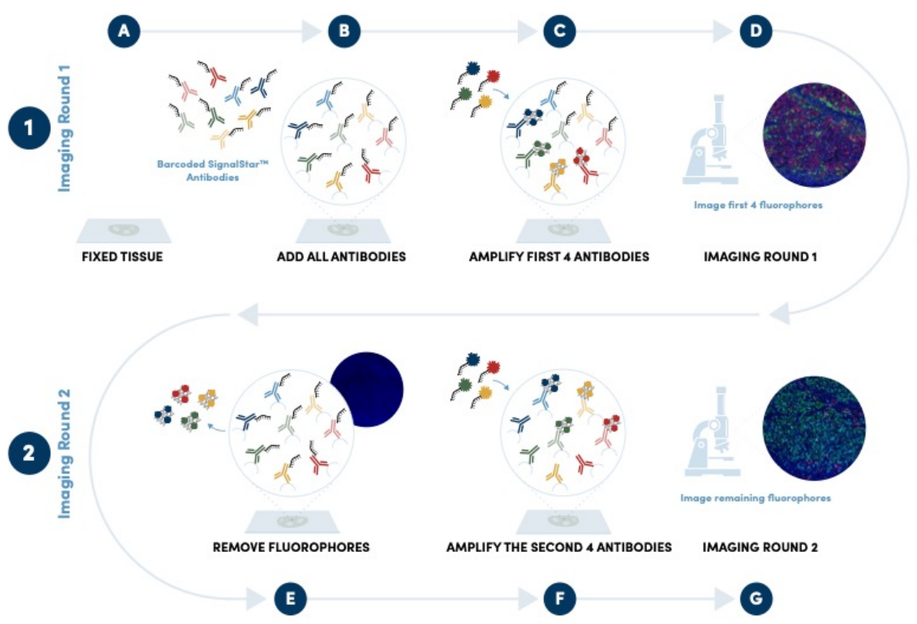

Figure 1. How SignalStar Works. The SignalStar assay allows for the simultaneous labeling of up to 8 targets in formalin-fixed paraffin embedded (FFPE) tissue. Deparaffinized and rehydrated FFPE tissue sections undergo antigen retrieval (A), and all antibodies in your plex-size of choice (3-8 maximum 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 full up to 8-plex image (G).

Figure 2. SignalStar staining matches chromogenic DAB in tissues with low, medium, or high target expression

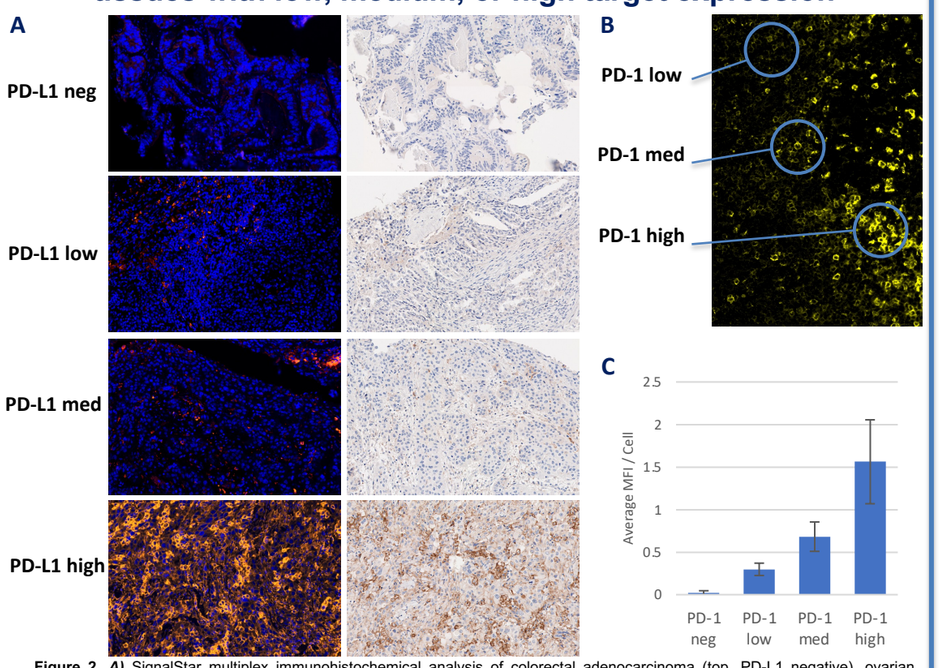

Figure 2. ***A)*** SignalStar multiplex immunohistochemical analysis of colorectal adenocarcinoma (top, PD-L1 negative), ovarian carcinoma (top middle, PD-L1 low), head and neck cancer (bottom middle, PD-L1 medium), and squamous cell carcinoma (bottom, PD-L1 high) using PD-L1 (E1L3N) & CO-0005-647 SignalStar™ Oligo-Antibody Pair #52085 (left column, orange) and ProLong Gold Antifade Reagent with DAPI #8961 (left column, blue) compared to chromogenic immunohistochemical analysis of a serial sections of paraffin-embedded tissues using PD-L1 (E1L3N®) XP® Rabbit mAb #13684 (right). ***B)*** SignalStar immunohistochemical analysis of paraffin-embedded human tonsil using PD-1 (Intracellular Domain) (D4W2J) & CO-0008-594 SignalStar™ Oligo-Antibody Pair #35347 (yellow). Staining was performed on the BOND RX autostainer by Leica Biosystems. ***C)*** Quantitation of the average mean fluorescence intensity (MFI) of PD-1 per cell. Quantitation was performed with inForm Tissue Analysis software from Akoya with thresholds as defined visually.

Figure 3. Reproducible results are obtained between 8-plex replicates and chromogenic DAB

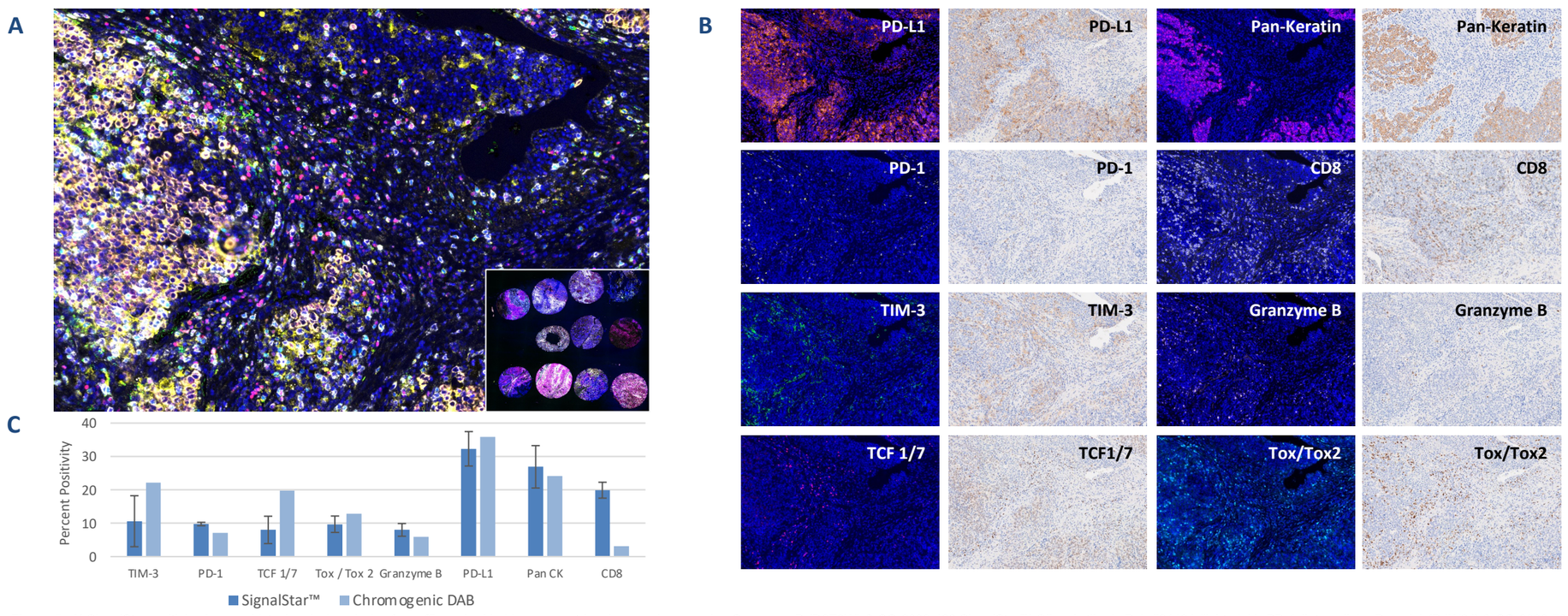

Figure 5. ***A)*** SignalStar multiplex immunohistochemical analysis of paraffin-embedded human gastric adenocarcinoma using Granzyme B (D6E9W) & CO-0009-488 SignalStar™ Oligo-Antibody Pair #84484 (light pink), PD-1 (Intracellular Domain) (D4W2J) & CO-0008-594 SignalStar™ Oligo-Antibody Pair #35347 (yellow), TCF1/TCF7 (C63D9) & CO-0006-647 SignalStar™ Oligo-Antibody Pair #25502 (red), Tox/Tox2 (E6I3Q) & CO-0016-750 SignalStar™ Oligo-Antibody Pair #81643 (cyan), TIM-3 (D5D5R™) & CO-00010-488 SignalStar™ Oligo-Antibody Pair #81365 (green), PD-L1 (E1L3N®) & CO-0005-594 SignalStar™ Oligo-Antibody Pair #28249 (orange), Pan-Keratin (C11) & CO-0003-647 SignalStar™ Oligo-Antibody Pair #16373 (magenta), CD8α (D8A8Y) & CO-0004-750 SignalStar™ Oligo-Antibody Pair #62750 (white), and ProLong Gold Antifade Reagent with DAPI #8961 (blue). 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 gastric adenocarcinoma using SignalStar antibodies described above (left) compared to chromogenic staining of serial sections of paraffin-embedded human gastric adenocarcinoma using Granzyme B (D6E9W) Rabbit mAb #46890, PD-1 (Intracellular Domain) (D4W2J) XP® Rabbit mAb #86163, TCF1/TCF7 (C63D9) Rabbit mAb #2203, Tox/Tox2 (E6I3Q) Rabbit mAb #73758, TIM-3 (D5D5R™) XP® Rabbit mAb #45208, PD-L1 (E1L3N®) XP® Rabbit mAb #13684, Pan-Keratin (C11) Mouse mAb #4545, and CD8α (D8A8Y) Rabbit mAb #85336 (right). ***C)*** Triplicate SignalStar 8-plex images were compared to the chromogenic DAB images. Percent positive cells in matching ROI from serial sections were quantified. Quantitation was performed with inForm Tissue Analysis software from Akoya with thresholds as defined visually.

Figure 4. SignalStar mIHC panels can be redesigned without additional optimization

A

Panel 1	Panel 2	Panel 3
Granzyme B:488	Granzyme B:488	Granzyme B:488
N/A	**PD-L1:594**	**LAG-3:594**
Pan CK:647	Pan CK:647	Pan CK:647
CD8:750	CD8:750	CD8:750
TIM-3:488	TIM-3:488	TIM-3:488
PD-1:594	PD-1:594	PD-1:594
TCF 1/7:647	TCF 1/7:647	TCF 1/7:647
Tox /Tox2:750	Tox /Tox2:750	Tox /Tox2:750

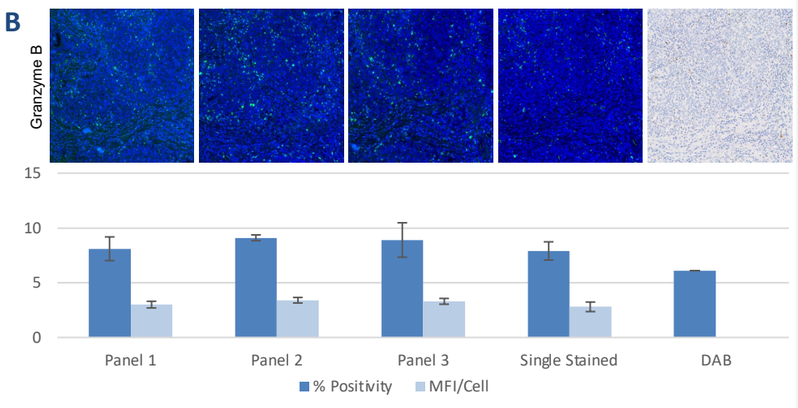

Figure 4. ***A)*** Three unique SignalStar panels were developed as indicated, swapping targets in the 594 channel of imaging round 1. Cells indicate target name : stain emission wavelength. ***B)*** SignalStar multiplex immunohistochemical analysis of serial sections of paraffin-embedded human gastric adenocarcinoma using Granzyme B (D6E9W) & CO-0009-488 SignalStar™ Oligo-Antibody Pair #84484 and ProLong Gold Antifade Reagent with DAPI #8961 (blue) in three unique SignalStar multiplex panels, compared to chromogenic immunohistochemical analysis of a serial section of paraffin-embedded human gastric adenocarcinoma using Granzyme B (D6E9W) Rabbit mAb #46890. ***C)*** Percent positivity and mean fluorescence intensity (MFI) per cell of Granzyme B in each SignalStar panel was compared to SignalStar single-plex staining and chromogenic DAB staining. Quantitation was performed with inForm Tissue Analysis software from Akoya.

Figure 5. SignalStar mIHC amplifies target signal

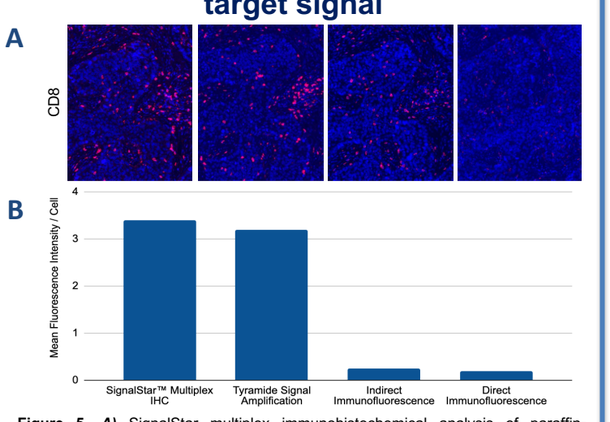

Figure 5. ***A)*** SignalStar multiplex immunohistochemical analysis of paraffin-embedded human gastric adenocarcinoma using CD8α (D8A8Y) & CO-0004-647 SignalStar™ Oligo-Antibody Pair #66676 (red) and ProLong Gold Antifade Reagent with DAPI #8961 (blue), compared to tyramide signal amplification, indirect immunofluorescence and direct immunofluorescence. ***B)*** Mean fluorescence intensity (MFI) per cell was compared across indicated mIHC modalities.

Figure 6. SignalStar assay enables identification and co-localization of cell populations

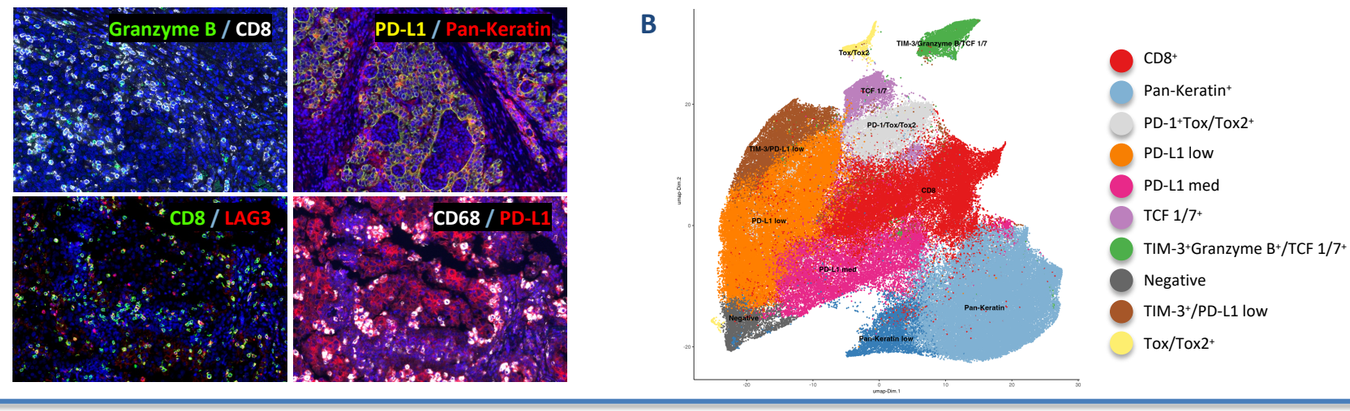

Figure 6. ***A)*** SignalStar immunohistochemical staining of paraffin-embedded human gastric carcinoma tissue using Pan-Keratin (C11) & CO-0003-647 SignalStar™ Oligo-Antibody Pair #16373 (top right, red), PD-L1 (E1L3N) & CO-0005-594 SignalStar™ Oligo-Antibody Pair #28249 (top right, yellow and bottom right, red), Granzyme B (D6E9W) & CO-0009-488 SignalStar™ Oligo-Antibody Pair #84484 (top left, green), CD8α (D8A8Y) & CO-0004-750 SignalStar™ Oligo-Antibody Pair #62750 (top left, white and bottom left, green), LAG3 (D2G4O™) & CO-0026-594 SignalStar™ Oligo-Antibody Pair #34308 (bottom left, red) and CD68 (D4B9C) & CO-0007-750 SignalStar™ Oligo-Antibody Pair #91044 (bottom right, white). ProLong Gold Antifade Reagent with DAPI #8961 (all images, blue) was used as a nuclear stain. All fluorophores have been assigned a pseudocolor, as indicated. Staining was performed on the BOND RX autostainer by Leica Biosystems. ***B)*** Cell type identification using Leiden clustering from the Giotto R package. Several cell types were identified including cytotoxic T cells (CD8+) and epithelial cells (Pan-Keratin+). Each cell is represented as a pair of UMAP coordinates.

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