**Background**
As multiplexed imaging has emerged as a powerful tool in spatial biology, there is a need for a robust, flexible, and easy-to-use assay to better understand the spatial context of the tumor microenvironment. SignalStar™ Multiplex Immunohistochemistry (IHC) technology from Cell Signaling Technology is a novel oligonucleotide (oligo)-based multiplex IHC assay that allows for the detection of up to 8 targets in FFPE tissue.

**Methods**
Antibodies used in the SignalStar multiplex IHC assay underwent CST’s first in class IHC-P validation process to ensure appropriate sensitivity and specificity. This includes testing relevant models with ranged target expression and orthogonal approaches, such as mass spec, in order to confirm consistent, reliable, and quantifiable results. CD11c, SIRPa, CD163, CD206, CD68, CD45, HLA-DRA, and Pan-Keratin antibodies were conjugated to oligos, then validated in the SignalStar Multiplex IHC assay to assess the myeloid compartment of the tumor microenvironment. Using the SignalStar assay, all antibodies are applied in cocktail in one incubation step. A network of complementary oligos with fluorescent channels 488, 594, 647, 750 amplify the signal of 4 oligo-conjugated antibodies in the first round of imaging. After imaging, fluorophores are removed from round 1 and amplification of 4 additional antibodies in the second round of imaging is performed. The antibodies were quantitatively validated in the SignalStar assay to ensure maximum fluorescent signal with minimal background, and compared against the chromogenic gold standard. Each antibody was validated across fluorescent channels, multiple rounds of imaging, and with manual vs automated protocols. The images were aligned using open-source software, and the signal of each target within the panel was quantified with respect to frequency and co-localization.

**Results**
The SignalStar technology demonstrates consistent signal compared to the chromogenic staining in serial sections across replicates. The technology shows flexibility in the channel and order in which each target is imaged. The percentage of positive cells for SIRPa was used to ensure target expression levels were consistent across fluorescent channels, amplification rounds and method of staining (manual vs automated). Co-localization of HLA-DRA and CD11c was quantifiable and compared to co-localization of HLA-DRA and Pan-Keratin. We were able to visualize and quantify specific phenotypes such as CD206+, CD68+, CD163+, and SIRPa+ cells using the SignalStar assay.

**Conclusions**
SignalStar multiplex IHC technology allows for the generation of consistent, reproducible and quantifiable phenotypic data that is vital when interrogating the immune tumor microenvironment. SignalStar produces a strong, robust signal that has been shown to match the chromogenic gold standard.

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