

Characterize the Immunosuppressive Tumor Microenvironment Using Multiplex IHC

Introduction

The clinical validation of immune checkpoint inhibitors as immunotherapeutic agents for a variety of cancers has revolutionized the field of cancer therapy. While significant improvement in patient outcome has been observed with previously untreatable tumors, not all patients respond to these drugs (1). An advanced understanding of the immune regulatory context of the tumor microenvironment (TME) is required to harness the power of the antitumor immune response. This will allow identification of novel therapeutic targets and potential biomarkers that can predict response to therapy (2,3).

Spatial localization, co-localization, and proximity of multiple biomarkers are critical when cataloging subsets of immune infiltrate and cancer cells and their interactions in the TME. Fluorescent multiplex immunohistochemistry (mIHC) assays enable identification of cellular phenotypes while simultaneously characterizing the protein expression of multiple therapeutic targets and predictive biomarkers in limited and valuable patient samples. For these reasons, mIHC, which facilitates detection of 6 or more proteins/biomarkers in formalin-fixed, paraffin-embedded (FFPE) tissue samples, is a valuable tool for immuno-oncology. CST offers highly specific and validated antibodies for IHC, including both mIHC and single/dual labeling chromogenic IHC, enabling investigators to get more information about relevant protein expression, localization, and proximity in context of the TME.

This application note explores the protocol and technical considerations for selecting and using antibodies in mIHC to assess immunosuppression mediated by myeloid cells in FFPE tissue samples.

Background and Results

Infiltrating myeloid cells constitute a significant component of the suppressive TME and possibly contribute to therapeutic failures (4). Myeloid cells can suppress the antitumor immune response through several mechanisms that can be therapeutically targeted. Recently, methods aimed at abolishing the suppressive activity of myeloid cells *in vivo* have been developed as potential therapeutic interventions in cancer (5). These include inhibitors against Arginase-1, IDO (6), M-CSF Receptor (7), and SIRPα/SHPS1 (8), as well as activators of CD40 signaling (9) among others.

The TME can contain a heterogeneous population of myeloid cells, including monocytes, tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), tumor-associated dendritic cells (TADCs), and tumor-associated neutrophils (TANs) that are defined by unique surface marker expression (10). These cell types are further characterized by different polarization states. Activated Type 1 myeloid cells stimulate T cell mediated antitumor immunity via secretion of pro-inflammatory cytokines, such as TNF- α , IL-1, IL-6, and IL-12. Additionally, they possess the capacity to process and present tumor antigens (10). Production of iNOS by these cells further enhances their ability to destroy tumors (11). In contrast, activated Type 2 myeloid cells are tolerogenic, mediating immunosuppression by secreting IL-10 and TGF- β , and

expressing enzymes Arginase-1 and IDO, which deplete the TME of nutrients essential for T cells (10, 12). High levels of inflammatory cytokines, such as IFN- γ , produced by tumors and infiltrating immune cells can also induce expression of PD-L1 and PD-L2 in myeloid cells, which suppresses anti-tumor immune responses mediated by CD8+ T cells expressing PD-1 (13).

A striking feature of myeloid cells is their plasticity, which makes it challenging to characterize their phenotype and function, and to study them outside their native environment (10). Therefore, multiple functional markers are required to allow the characterization of a cell as being more immunosuppressive or immunostimulatory. In an effort to characterize the immunosuppressive myeloid compartment of the TME, an mIHC panel of antibodies detecting IDO, Arginase-1, PD-L1, PD-L2, CD68 and cytokeratin (CK) (**Table 1**) was applied to FFPE-tissue sections of non-small cell lung cancer (NSCLC) (**Figure 1**) and ovarian carcinoma (**Figure 2**).

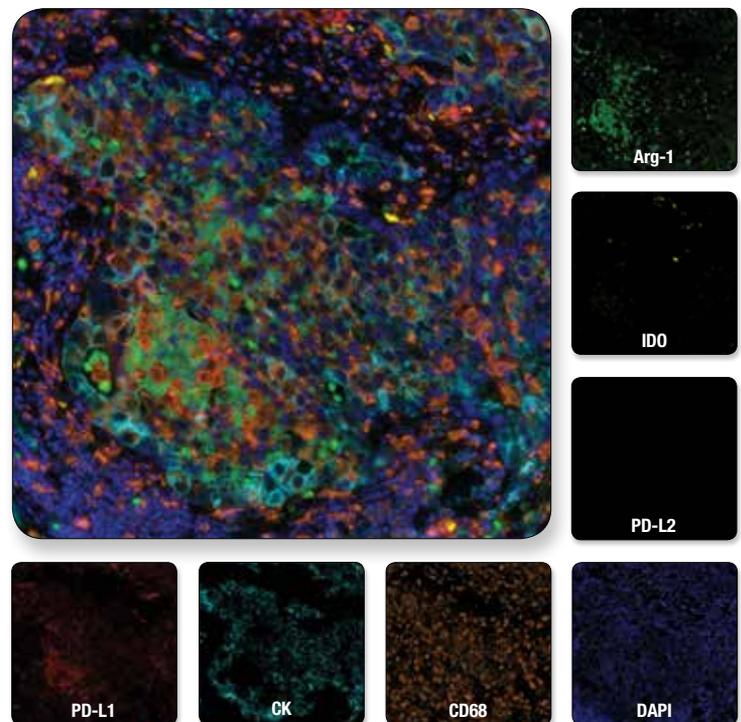


Figure 1: Multiplex IHC analysis of NSCLC probed with a 6-plex panel labeling markers of immunosuppression in the TME. PD-L2 was absent in this field, as observed by the lack of signal in its individual channel.*

*PD-L2 is not expressed in all tumors; only about 20% of NSCLC patients express PD-L2 (Pinato DJ, et al, Oncoimmunology 2016).

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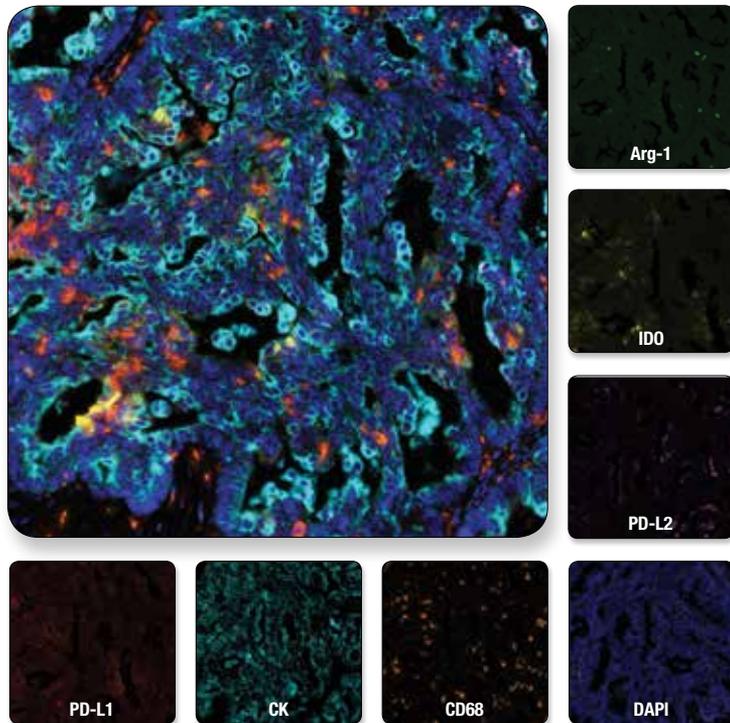


Figure 2: Multiplex IHC analysis of ovarian carcinoma probed with a 6-plex panel labeling markers of immunosuppression in the TME.

Table 1: Immunosuppressive Myeloid Cell Antibody Selections

ORDER	TARGET	CST ANTIBODY	DILUTION	FLUOROPHORE
1st	PD-L2	PD-L2 (D7U8C™) Rabbit mAb #82723	1:100	Alexa Fluor® 594
2nd	IDO	IDO (D5J4E™) Rabbit mAb #86630	1:15,000	Alexa Fluor® 555
3rd	Arginase-1	Arginase-1 (D4E3M™) XP® Rabbit mAb #93668	1:5000	FITC
4th	PD-L1	PD-L1 (E1L3N®) XP® Rabbit mAb #13684	1:1400	Cy™5
5th	CD68	CD68 (D4B9C) XP® Rabbit mAb #76437	1:6000	Cy™5.5
6th	CK	Pan-Keratin (C11) Mouse mAb #4545	1:50	Alexa Fluor® 350

Methods

The tyramide-based mIHC method employs a serial labeling strategy (**Figure 3**). Following incubation of tissue with primary antibody, HRP-conjugated secondary antibodies catalyze deposition of tyramide-fluorophore conjugates. The fluorophore-conjugated tyramide remains covalently bound to the tissue while the antibodies are removed via microwave treatment between labeling. Note that the removal of primary antibody after each labeling round allows for the use of multiple antibodies from the same host species (e.g., rabbit).

FFPE tissue section is processed and analyzed for mIHC in accordance with the protocol.

- 1. Deparaffinization/Rehydration:** To prepare for antigen retrieval, tissue section was deparaffinized with xylene, followed by rehydration with 100% ethanol, 95% ethanol, and dH2O.
- 2. Antigen Retrieval:** Extensive optimization was performed to ensure maximal unmasking of each epitope to allow for efficient binding of the primary antibody.
- 3. Antibody Titration:** The optimal dilution for each primary antibody was determined empirically to ensure maximal fluorescence intensity and minimal background signal for each target of interest.
- 4. Staining:** Incubation with primary antibodies diluted in SignalStain® Antibody Diluent #8112 was performed under humidified conditions at room temperature. Subsequent incubation with either SignalStain® Boost IHC Detection Reagent (HRP, Mouse) #8125 or SignalStain® Boost IHC Detection Reagent (HRP, Rabbit) #8114, as appropriate, was performed.
- 5. Image Acquisition/Analysis:** The Mantra® Quantitative Pathology Workstation system was used for multispectral imaging. Image analysis was performed using the InForm® Image Analysis software package.

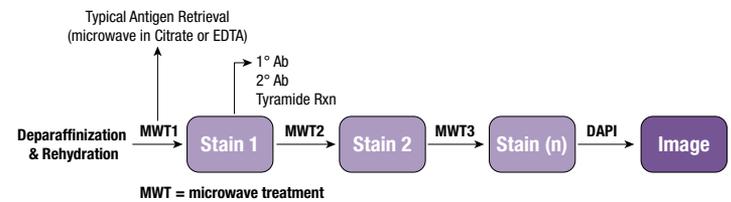


Figure 3: Schematic representation of the fluorescent mIHC workflow.

Optimization

Titration: Prior to performing a multiplex experiment, the optimal concentration of each primary antibody needs to be determined in a singleplex setting. Using FFPE ovarian carcinoma tissue sections, the optimal dilution of IDO in the singleplex setting was found to be 1:200 with respect to both maximal fluorescence intensity and the peak signal to noise (S/N) ratio. However, in a multiplex setting when stained first with PD-L2 followed by staining with IDO at 1:200, a bleed over of the IDO signal was observed in the PD-L2 channel (IDO signal is present in both IDO and PD-L2 channels). To resolve this, an extended titration of IDO was performed in the Alexa Fluor® 555 channel. The dilution at which both IDO and PD-L2 signals were distinct in separate channels was determined to be 1:15,000. The bleed-through of the IDO signal is illustrated in (**Figure 4**). When stained with IDO alone, a bleed over of the IDO signal (Alexa Fluor® 555) into the PD-L2 channel (Alexa Fluor® 594) is evident only in the tissue stained at 1:200 and not in the tissue stained at 1:15,000. We highly recommend applying this approach to establish the optimal dilutions for all antibodies to be used in a multiplex experiment.

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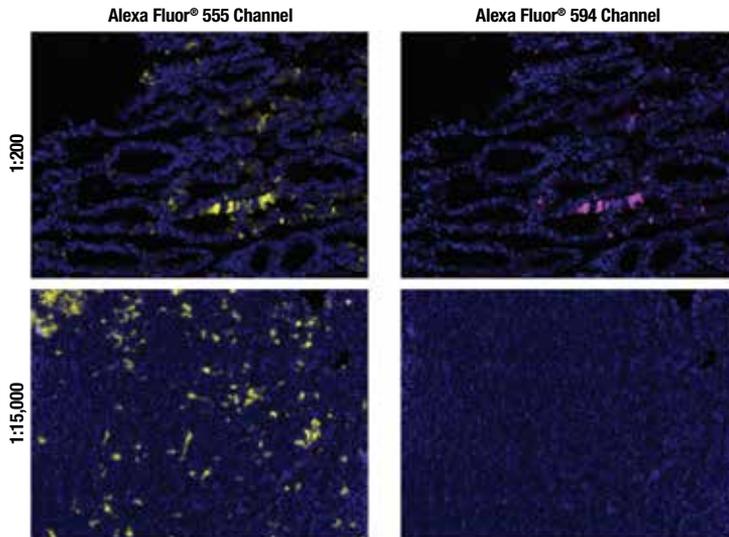


Figure 4: FFPE ovarian carcinoma tissue was stained with IDO at 1:200 (top) and 1:15,000 (bottom) followed by detection via tyramide-Alexa Fluor[®] 555.

Order Optimization: The order in which antibodies in a multiplex panel are applied to a tissue section must be optimized to ensure that multiple rounds of heating do not compromise the integrity of the epitope of interest. Note that for the purposes of order optimization each tissue section was labeled/stained only once and subjected to microwave treatment the same number of times irrespective of labeling order. IDO (D5J4E[™]) Rabbit mAb #86630 exhibits a reduction of signal dependent on labeling order, likely due to epitope loss (**Figure 5**). Our protocols include IDO labeling in the second step of the suppressive myeloid cell panel (**Table 1**). The positioning of an antibody is ultimately determined after performing this optimization with all antibodies to be included in the panel such that all signal intensities are balanced when the antibodies are combined.

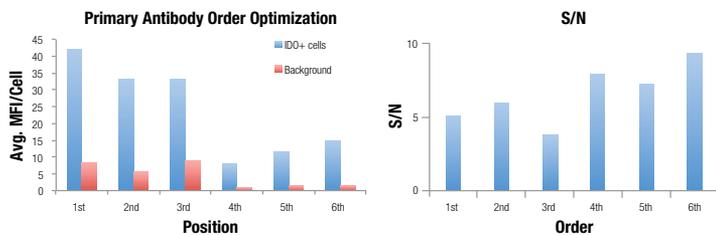


Figure 5: Mean fluorescence intensity (MFI) as a function of IDO (D5J4E[™]) Rabbit mAb #86630 labeling order (position).

Fluorophore Pairing: The objective of this optimization step is to achieve balanced signal intensities within the panel such that the fluorescence signal originating from targets of high abundance does not drown out the signal originating from targets of lower abundance. To this end, it is good practice to pair antibodies detecting weakly expressed targets with the brightest fluorophores (**Figure 6**). We recommend analyzing a matrix composed of optimized primary antibodies paired with each available fluorophore in order to achieve an optimal balance with respect to both signal intensity and S/N ratio.

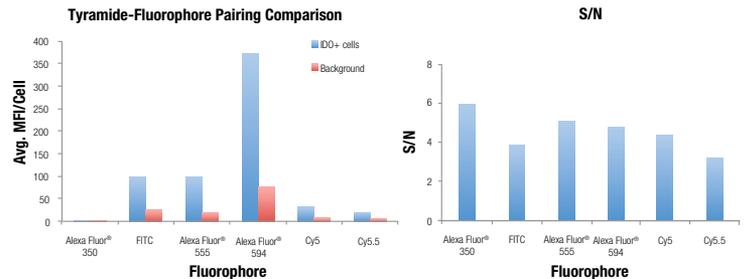


Figure 6: Mean fluorescence intensity (MFI) of IDO (D5J4E[™]) Rabbit mAb #86630 as a function of tyramide-fluorophore conjugate.

Conclusion

mIHC enables in-depth characterization of the tumor microenvironment. The data shown here demonstrate the capability to image 6 targets plus DAPI in FFPE tissue samples. Rational optimization of experimental parameters including antibody dilutions, order of staining, and fluorophore pairing is recommended when designing mIHC panels. Further analysis of biomarker expression patterns, for example co-expression or mutual exclusivity in the same cell or in adjacent cells, is also possible with mIHC. Fluorescent mIHC embraces the complexity of the immunosuppressive myeloid cell compartment by enabling the comprehensive phenotyping of immune cells and therapeutic targets. Comprehending the role of myeloid cells in the anti-tumor immune response will facilitate development of tailored and combinatorial therapeutic interventions.

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