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Sequential Multiplex Immunofluorescence Technology (SMIFT): A New Staining Strategy for Immunotherapy

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Introduction

Tumor tissues exhibit a multitude of different cell types that have extravasated from other regions in the body. These cells are likely to represent heterogeneous populations of immune and effector cells.[1] Assessing and quantifying immunotherapy markers in tissues such as melanoma or squamous cell carcinoma could be of significant value for patient treatment. An innovative method that will enable the sequential application of three or more mouse monoclonal antibodies (MMAs) has been developed. This method allows the detection of multiple immune cell markers on a single tissue section. Previous methods using a sequential staining technique of MMAs required manual application and time-consuming heating and stripping between steps.[2,3] The assessment of multiple targets can now be determined in less than eight hours using full automation without heating or stripping procedures. This method may be valuable when the quantification of specific immunotherapy markers is required.

Materials and Methods

Two assays were developed using Sequential Multiplex Immunofluorescence Technology (SMIFT): FOXP3, CD163, PD-1, CD8 as well as p40, Desmoglein 3 (DSG3), TTF-1, CD8. Samples of tonsil, cutaneous SqCC, lung SqCC and melanoma were fixed in 10% NBF for 24 hours and processed in paraffin. Sections were cut at 5 microns and adhered to glass slides. Sections were deparaffinized, peroxidase blocked, and antigen retrieved in a citrate-based buffer at pH 6.0 within a pressure cooker at 95°C for 40 minutes. All antibodies were diluted in diluent and applied to tissues to determine optimal IF (and IHC) titers. IHC detection of each antibody was accomplished with an anti-mouse/anti-rabbit HRP polymer followed by goat anti-mouse fluorophores 594, 488 or 421 and goat anti-rabbit fluorophores 546 or 480 for IF. An in-house blocking reagent was incorporated into the protocol to prevent cross-reactivity during multiple detection of fluorophores. Both assays were comprised of two sequential MMAs followed by a third application of a mouse/rabbit monoclonal antibody cocktail to complete the procedure. Sections were then counterstained in DAPI (or hematoxylin). intelliPATH FLX® and ONCORE™ automated staining platforms as well as reagents, including primary antibodies, were supplied by Biocare Medical; detection fluorophores were obtained from Thermofisher Scientific and Jackson ImmunoResearch Laboratories.

Table 1

Sequential multiplexing technology (SMIFT) approach	Time
Deparaffinize slides	25 min.
Perform Antigen Retrieval at 95°C	40 min.
Apply first primary antibody (FOXP3 or p40)	60 min.
Detect with first anti-mouse fluor (594)	30 min.
Apply blocking agent	15 min.
Apply second mouse antibody (CD163 or DSG3)	60 min.
Detect with second anti-mouse fluor (488)	30 min.
Apply blocking agent	15 min.
Apply cocktail of third mouse antibody + rabbit antibody (PD-1 mse + CD8 rab or TTF-1 mse + CD8 rab)	60 min.
Detect with cocktail of anti-mouse fluor (546 or 421) + anti-rabbit fluor (480 or 546)	30 min.
Counterstain nuclei with DAPI	3 min.
Mount sections with anti-fade and view with appropriate filters	
Total time	*~ 7 hrs.*

*Sections were washed between steps in TBS.

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Table 2

Antibodies tested with SMIFT on tonsil, cutaneous SqCC, lung SqCC and melanoma

Antibody	Application Order	Species	Clone	Cellular Target	Flours Tested	Color
p40	First	mse	BC28	SqCC	488/594	Green/Red
FOXP3	First	mse	236A/E7	T-regulatory cells	594	Red
DSG3	Second	mse	BC11	SqCCs	594/488	Red/Green
CD163	Second	mse	10D6	TAMs*	488	Green
PD-1	Third (cocktail)	mse	NAT105	Activated B and T cells	546/421	Orange/Blue
TTF-1	Third (cocktail)	mse	SPT24	ADC**	546	Pseudocolored Yellow
CD8	Third (cocktail)	rab	SP16	Cytotoxic T cells	480/546	Pseudocolored Purple/Orange

*Tissue associated macrophages

**Adenocarcinoma

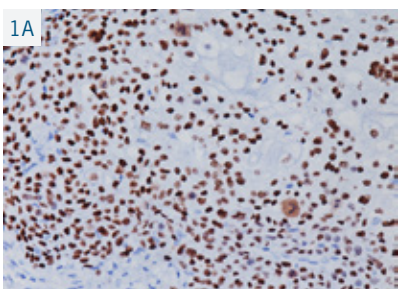
Results

Individual staining results for each antibody target used in this study were first determined by IHC using a cutaneous SqCC sample (Figure 1). Staining intensities for each target were 3+ (except TTF-1 as TTF-1 was not present). Initial IF experiments determined that a sequential approach with same species antibodies (mouse) followed by anti-mouse fluorophores generated an expected color merge (Figure 2A). A blocking step post-fluorophore detection was necessary to prevent this effect (Figure 2B). This approach was utilized for both assays in this study and equally effective for two sequential mouse primary incubations/detections followed by a third mouse plus rabbit antibody cocktail and detection (Figures 3, 4D, 4H and 6E). Staining intensities were further compared to individual antibody formats for both assays. Staining of each target was clearly identified when multiplexed. However, the presence of one target (e.g. FOXP3) was minimal in some tissues studied, thus making it difficult to identify within a field of view (Figure 4D).

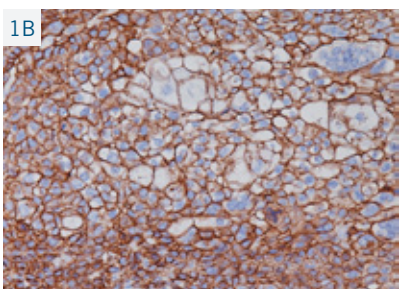
Fluorescence intensities were consistently 3+, scored on a subjective scale from 1 to 3+ for each target fluorophore when read at 20X. Each channel corresponding to individual antibody targets on tonsil, cutaneous SqCC, lung SqCC and melanoma were captured and, then, merged via imaging software to form multiplexed images.

The significance of multiplexing can readily be identified when comparing channels for PD-1 (546/orange) and CD8 (480/purple) in tonsil versus melanoma. Tonsil reveals anticipated compartmental staining for PD-1 (orange) in the germinal center and CD8 (purple) in mantle zones. However, when channels are combined, PD-1 and CD8 appear in a near identical pattern in this melanoma sample, indicating a dual expression of these markers (Figure 4H).[4]

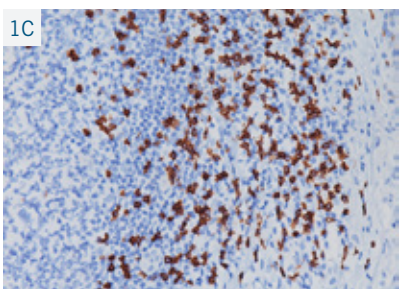
Figure 1: Cutaneous SqCC (A-F)



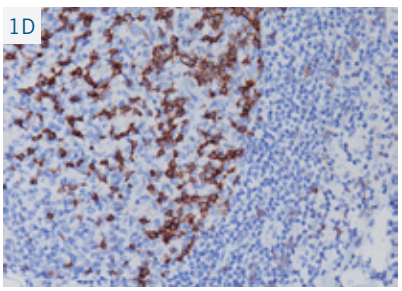
SqCC stained with p40



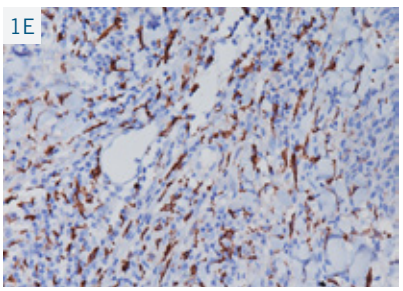
SqCC stained with DSG3



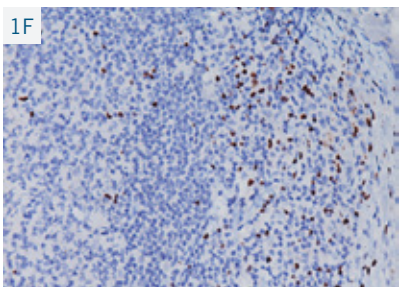
SqCC stained with CD8



SqCC stained with PD-1

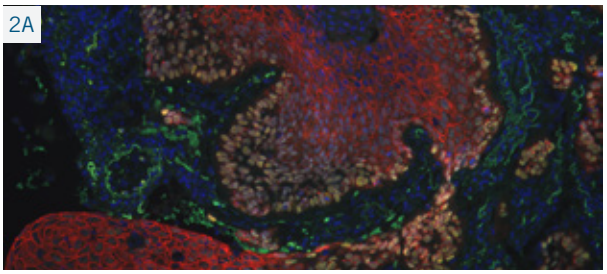


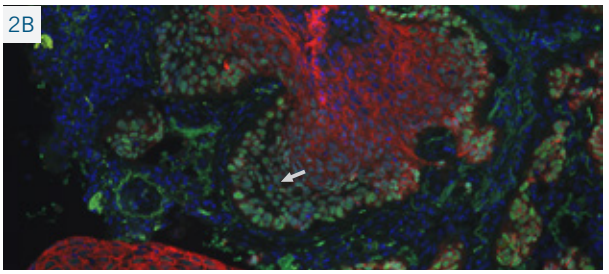
SqCC stained with CD163



SqCC stained with FOXP3

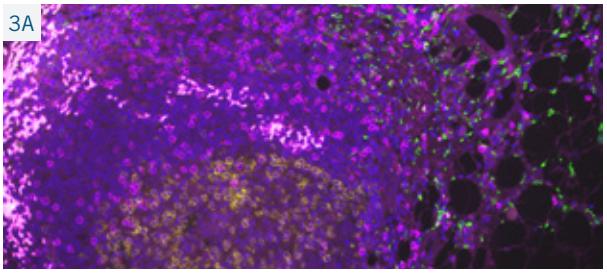
Figure 2: Lung SqCC (A-B)



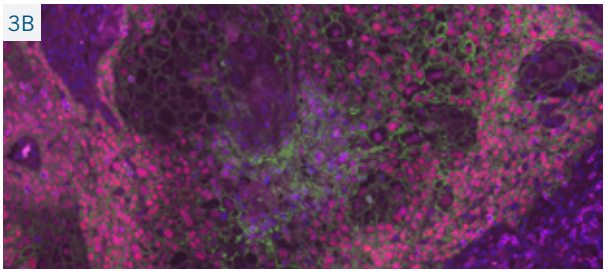


Blocking protocol demonstrated in lung SqCC with mouse p40 detected by anti-mouse 488 (green) followed with mouse DSG3 detected by anti-mouse 594 (red). Colors do not merge (arrow)

Figure 3: Cutaneous SqCC (A-B)

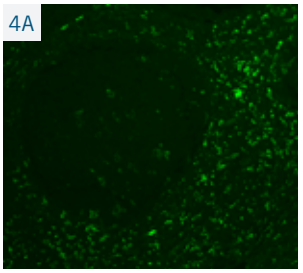


SMIFT: Cutaneous SqCC stained with FOXP3 (594/red - negative), CD163 (488/green), PD-1 (546/pseudocolored yellow), CD8 (480/pseudocolored purple) and DAPI.

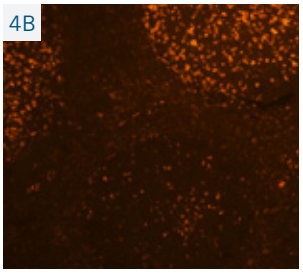


SMIFT: Cutaneous SqCC stained with p40 (594/red), DSG3 (488/green), PD-1 (546/orange - negative), CD8 (480/pseudocolored purple) and DAPI.

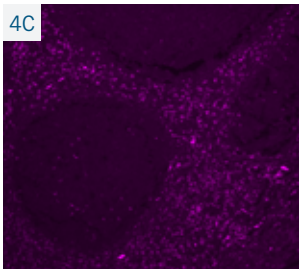
Figure 4: Tonsil control (A-D) and melanoma (E-H)



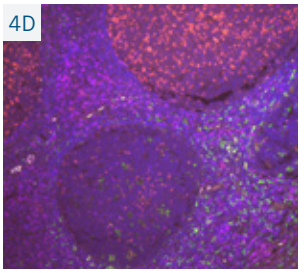
Tonsil stained with CD163 (488/green)



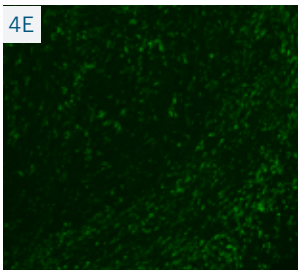
Tonsil stained with PD-1 (546/orange)



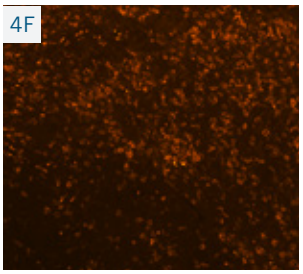
Tonsil stained with CD8 (480/pseudocolored purple)



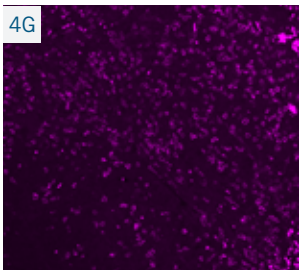
SMIFT: Tonsil stained with FOXP3 (594/red - minimal), CD163, PD-1, CD8 and DAPI.



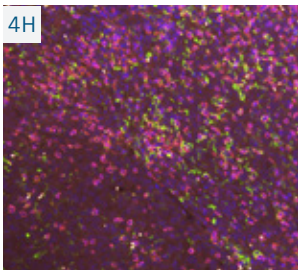
Melanoma stained with CD163 (488/green)



Melanoma stained with PD-1 (546/orange)

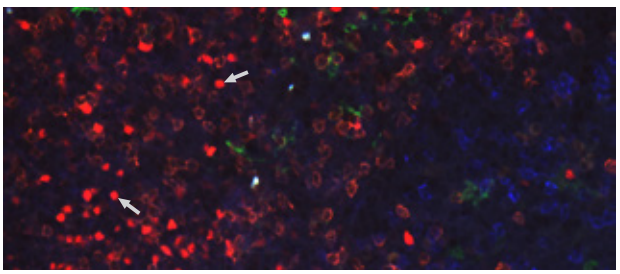


Melanoma stained with CD8 (480/pseudocolored purple)



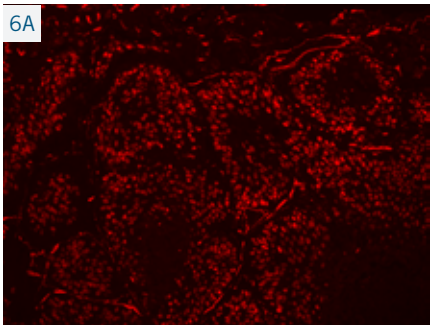
SMIFT: Melanoma stained with FOXP3 (594/red - minimal), CD163, PD-1, CD8 and DAPI.

Figure 5: Tonsil

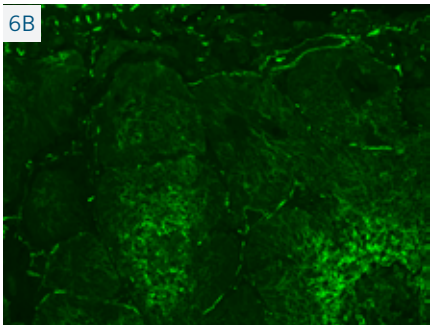


SMIFT: Tonsil mantle zone stained with FOXP3 (594/red - arrows), CD163 (488/green), PD-1 (421/blue) and CD8 (546/orange).

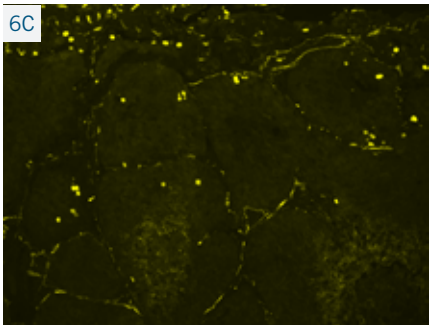
Figure 6: Lung SqCC (A-E)



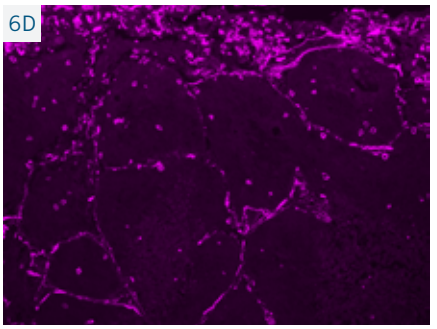
Lung SqCC stained with p40
(594/red)



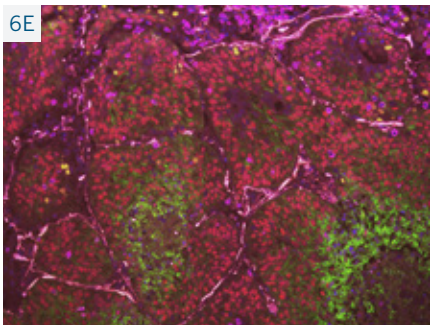
Lung SqCC stained with DSG3
(488/green)



Lung SqCC stained with TTF-1
(546/pseudocolored yellow)



Lung SqCC stained with CD8
(480/pseudocolored purple)



SMIFT: Lung SqCC stained with p40, DSG3, TTF-1, CD8 and DAPI.

Discussion

The SMIFT approach demonstrates that multiplexing of immunotherapy markers is a feasible method in tonsil, cutaneous SqCC, lung SqCC and melanoma. This is important when identifying CD163 tumor associated macrophages (TAMS), effector T or B-cells (e.g. CD8/PD-1) or other cell populations known to influence patient outcome based on the presence of these cells within the tumor microenvironment. Quantification along with ratio determination of cell types within a tumor has been shown to provide a guide for potential immune therapy intervention.[5] Although limited tumor samples were evaluated in this study, each antibody was capable of clearly recognizing its target for both IHC and IF. Using tonsil as a model was an essential first step in this process since all markers (except TTF-1) were present to some degree and in close proximity to each other, thus making it simple to assess fluorophores within a field of view. Further SMIFT testing and validation in malignant tissues via TMAs with additional immunotherapy panels to determine sensitivity and specificity is required before such an approach can be applied in a diagnostic and/or prognostic fashion.[6]

Conclusion

Multiplexing can be achieved with a newly developed method termed “SMIFT”, a sequential and multiplex IF approach to identify and quantify multiple targets on a single tissue section. Three sequential applications of four antibodies can be performed in less than eight hours on the bench or with full automation. This is in contrast to previous overnight methods. Most importantly, this approach could be used to assess additional immunotherapy markers in different tissues and may provide prognostic or predictive value.

References

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