Bringing the Tumor Microenvironment into Focus: Simplified Development of Seven-Color Multiplex Immunohistochemistry-Immunofluorescence (mIF) Panels

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Highlights

- Multiplex immunohistochemistry-immunofluorescence (mIF) panel development enables simultaneous detection of multiple proteins of interest within a single sample, advantageous in analysis of limited tissue such as tumor biopsy.
- The applications of mIF span clinical and basic research, but a 7-color mIF can take ≥8 weeks to develop.
- Here, a faster, simplified approach to 7-color mIF panel development is described with a focus on the tumor microenvironment.

Introduction to mIF

Recent advances in multiplex immunohistochemistry (IHC) and multispectral imaging facilitate simultaneous analysis of multiple tissue markers within a given sample using an mIF panel. mIF has several advantages relevant to both clinical and basic research applications including 1) conservation of limited sample by visualizing multiple targets within a single tissue section; 2) preservation of tissue architecture and capture of microenvironment data; 3) provision of data regarding colocalization and spatial orientation of proteins; 4) detection of low-level binding sites and quantitation of target intensity; 5) simplified panel design, wherein any primary antibody validated for IHC, regardless of host species, can be utilized for each target of interest so long as a primary-specific secondary antibody is used; and 6) use of a DAPI (4′,6-diamidino-2-phenylindole) counterstain.

mIF is performed on formalin-fixed paraffin-embedded tissue sections, where fluorescent detection dye is covalently attached to the tissue allowing primary/secondary antibodies to be removed for successive rounds of staining (Figure 1). Briefly, the primary and secondary antibodies corresponding to the first target of interest are deposited and incubated with the fluorescent detection substrate. The antibodies are removed from the tissue using heated washes (heat-induced epitope retrieval [HIER]), and the staining process is repeated for subsequent target(s) of interest until all targets have been labeled. Substrate is covalently bonded to the target protein site when tyramide forms bonds with tyrosine residues on or near the antigen and the fluorophore is permanently deposited at the site of the antigen. Multiple rounds of staining are allowed by the process of stripping primary/secondary antibody pairs while preserving antigen-associated fluorescent signal. Prior to imaging, a DAPI counterstain and coverslip is applied.

While a powerful technique, the real-world application of mIF is currently limited given the time required to develop an optimized panel. The following method was applied to accelerate and simplify the development of 7-color mIF panels.

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Method for development of a faster, simplified 7-color mIF panel

Formalin-fixed, paraffin-embedded human tissue was stained with PathPlex™ Panel 4 IHC-validated primary antibodies (Bethyl Laboratories [A810-004]), mouse or rabbit HRP-conjugated secondary antibodies (Bethyl Laboratories [A90-116P, A120-501P]), and detected using Opal™ Polaris 7-color IHC kit fluorophores (Perkin Elmer [NEL861001KT]). Primary antibody order was optimized utilizing tissue microarray serial sections (3 slides per target) by staining after the first, third, or sixth HIER. Slides were imaged using the same exposure time and analyzed for target nucleus counts, signal intensity, and background. Primary antibody order in the 7-color mIF was confirmed using a single stain. Whole slide scans were generated using the Vectra Polaris® and analyzed with the InForm® image analysis package.

Results

Using optimal primary antibody dilution and application order (Figure 2A; antibody order Option 1 and Option 2), 7-color mIF development time was reduced using IHC-validated antibodies. The number of slides was reduced from a possible 720 combinations to 3 per target (18) plus confirmation 7-color slides. Primary antibody order (confirmed on both tonsil and cancer array cores) was guided by the ratio of target staining/DAPI nuclear counts, average intensity, and overall background of the 3 slides (Figure 2). Certain targets (ie, FOXP3) revealed optimal staining with greater intensity and lower background when stained first, whereas the opposite, or a lack of effect of multiple HIER, may apply to other targets. Optimal primary antibody order was determined to be Option 1, resulting in a panel containing CD3, cytokeratin, CD8, CD68, PD-L1, and FOXP3 (Figure 3).

Conclusion and broader application to clinical and basic research

mIF is a powerful technique that allows for examination of spatial arrangement of proteins of interest as well as protein interactions/co-localization of multiple targets within a single tissue specimen. Seven-color mIF panels can take ≥8 weeks to optimize, but use of high-quality IHC-validated antibodies whose order of staining has been pre-determined has immense time- and resource-saving potential.

To create a custom 7-color mIF panel, targets of interest and appropriate high-quality IHC-validated antibodies are selected, and optimal dilution confirmed. Panel order is determined by identifying the best signal using serial tissue sections and staining slides after the first, third, and sixth HIER. Resulting images are compared to determine optimal primary antibody order. Antibodies are paired with fluorophores such that antibodies to markers that may have overlapping signal are separated by ≥1 Opal, and order for mIF staining is confirmed.

Guidelines for the optimization of mIF, including a step-by-step protocol, can be found at https://www.bethyl.com/content/protocol-multiplexing. For further information regarding validated PathPlex™ antibodies, visit https://promotions.bethyl.com/multiplexing/.

Supporting Figures

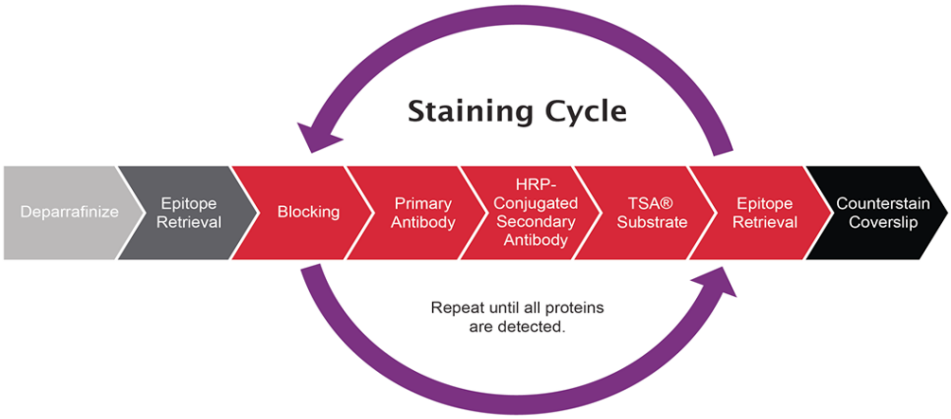
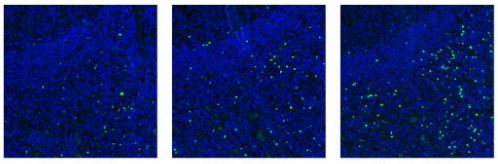


Figure 1. mIF staining cycle. Abbreviations: HRP, horseradish peroxidase TSA, tyramide signal amplification.

A.

Target (Bethyl Catalog No)	Best Signal		
	1st HIER	3rd HIER	6th HIER
CD68 A500-018A		X	X
Cytokeratin A500-019A	X	X	X
CD3E A700-016	X		
PD-L1 A700-020			X
FOXP3 A700-034			X
CD8A A700-044	X	X	X

B.



First HIER | Third HIER | Sixth HIER

C.

	1st HIER	3rd HIER	6th HIER
FOXP3	1684	2419	3281
DAPI+	35151	32876	33012
Ratio	5	7	10
Intensity	77	81	88
Background	-	-	-

Figure 2. Determination of optimal primary antibody order. (A) Using 3 slides per target, slides were stained after the first, third, and sixth heat-induced epitope retrieval (HIER) to determine the best signal for each primary antibody in the panel. Potential optimal primary antibody order was determined to be either: Option 1 of CD3, cytokeratin, CD8, CD68, PD-L1, and finally FOXP3, or Option 2 of CD3, cytokeratin, CD8, CD68, FOXP3, and finally PD-L1. **(B)** Serial section comparison of FOXP3 staining after the first, third, and sixth HIER. **(C)** InForm® image analysis of FOXP3 staining after the first, third, and sixth HIER. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole.

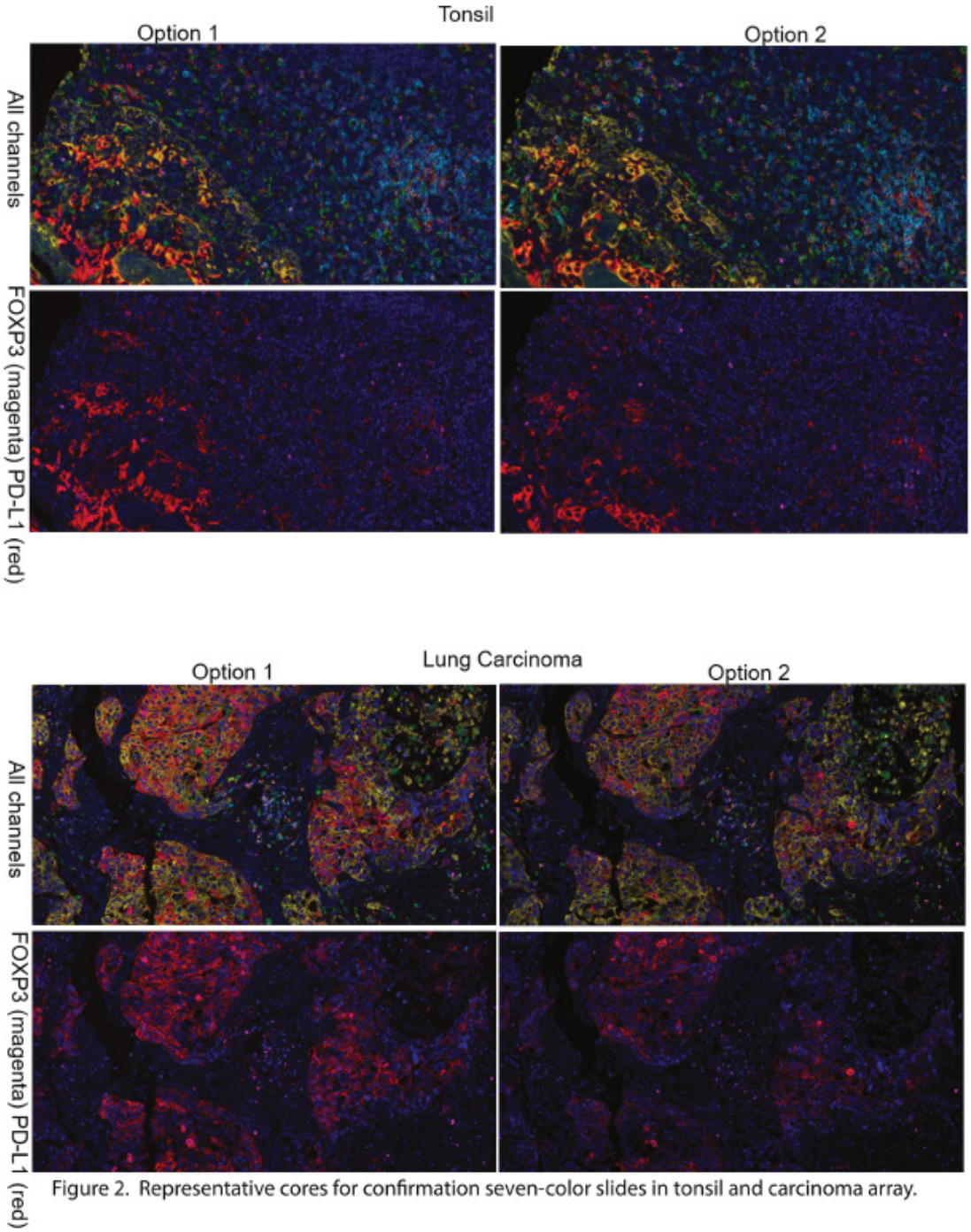


Figure 2. Representative cores for confirmation seven-color slides in tonsil and carcinoma array.

Figure 3. Representative cores for confirmation of 7-color slides in tonsil and lung carcinoma array. Possible optimal primary antibody order is depicted as Option 1 (CD3, cytokeratin, CD8, CD68, PD-L1, and finally FOXP3) and Option 2 (CD3, cytokeratin, CD8, CD68, FOXP3, and finally PD-L1). Option 1 was considered the optimal primary antibody order.

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