Multiplex Made Practical: A Direct-Conjugated Antibody Panel Design Workflow

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Abstract

This work highlights best practices for designing spatial biology multiplex immunohistochemistry (IHC) panels using directly-conjugated primary antibodies, which streamline workflows without amplification bias. We present a practical framework for building and optimizing panels to characterize immune and structural features in healthy colon and colon adenocarcinoma, including in-house antibody conjugation and strategies to reduce autofluorescence and improve spectral unmixing (imaged on the EVOS^{TM} S1000). Using a Colorectal Cancer Immuno-Oncology Panel targeting markers CD3, CD8a, CD68, Pan-CK, p53, Ki-67, and β-catenin, we observed distinct differences between healthy and tumor tissues, including reduced immune cell markers and uniform p53 expression in tumors. These results demonstrate that directly-conjugated multiplex panels enable a robust and scalable approach for resolving complex tissue architecture and generating high quality spatial insights into cancer biology.

Introduction

Multiplex IHC enables simultaneous visualization of multiple biomarkers in healthy and adenocarcinoma colon tissue, supporting detailed comparisons of cellular composition and tumor microenvironment changes that drive cancer progression and inform diagnostic and therapeutic strategies. The use of primary antibody cocktails streamlines the workflow, allowing rapid, one-step staining of up to eight targets plus DAPI shown below in **Figure 1**.

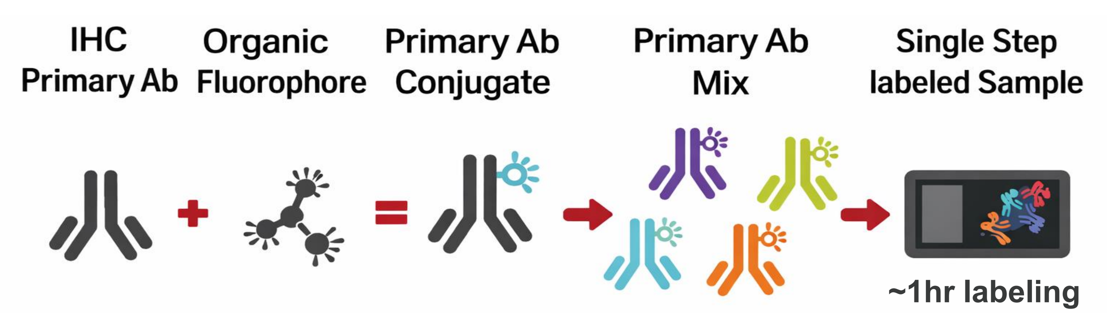

Figure 1. Primary antibody conjugation and staining workflow.

Panel design

Choose immunomarkers & assign fluorophores with care

A key first step in panel design **is selecting the most informative markers for your study objectives**. For this panel, the chosen markers collectively evaluate tumor proliferation (Ki-67), Wnt pathway activation (β-catenin), tumor suppressor status (p53), epithelial identity (Pan-CK), and the composition and immune contexture of the tumor microenvironment (CD3, CD8a, CD68, PD-1), enabling a comprehensive and integrated assessment of tumor biology and immune response.

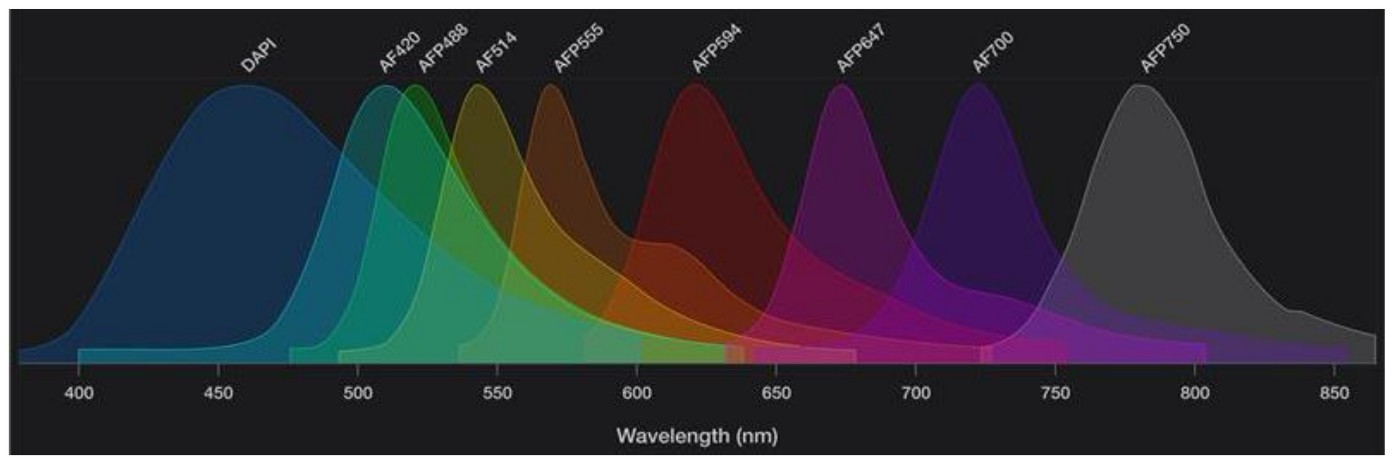

Figure 2. Emission spectra of Alexa Fluor™ dyes offered for primary antibody conjugates for spatial imaging (plus DAPI). Each dye has its own unique spectral fingerprint that must be taken into consideration when multiplexing.

Once markers have been chosen, selecting which fluorophores to use for each marker takes careful thought and time to plan. **Seven of the targets used in this experiment are available in the Thermo Fisher catalog. CD3, however, is not currently offered in the desired fluorophore, so it was conjugated to Alexa Fluor™ 514 using a ReadyLabel™ Antibody Labeling Kit. This kit enables straightforward labeling of IgG antibodies from most species, regardless of antibody concentration or storage buffer conditions.**

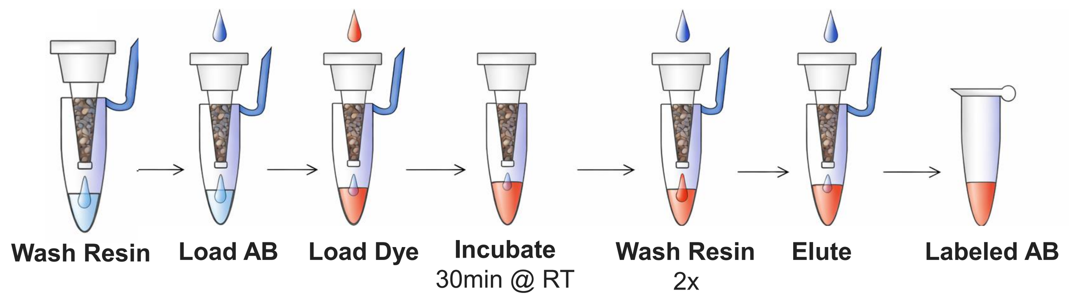

Figure 3. Primary antibody purification and conjugation using the ReadyLabel™ Antibody Labeling Kit workflow. **The kit comes with two low pH elution buffers, but we suggest using pH 2 for the best elution success across most antibodies.**

Primary antibody conjugate	Final conc.	Cell type/target	SKU
β-catenin Alexa Fluor™ 420	10 µg/mL	Epithelial/ adhesion cells	N/A
CD68 Alexa Fluor™ Plus 488	16 µg/mL	Macrophages	752068882
CD3 Alexa Fluor™ 514 (ReadyLabel conjugation)	1 µg/mL	Mature T cells	N/A
CD8a Alexa Fluor™ Plus 555	10 µg/mL	Cytotoxic T cells	754008582
p53 Alexa Fluor™ Plus 594	10 µg/mL	Cell cycle regulation	755800294
PD-1 Alexa Fluor™ Plus 647	1.6 µg/mL	Activated/ Exhausted T cells	756800394
Pan-CK Alexa Fluor™ 700	4 µg/mL	Epithelial cells	56900382
Ki-67 Alexa Fluor™ Plus 750	8 µg/mL	Proliferating cells	757569882

Table 1. Finalized marker panel for colorectal cancer immuno-oncology, with optimized concentrations for the primary antibody staining cocktail.

Three important guidelines to consider when multiplexing in IHC:

1. Strategically pair markers with fluorophores

In multiplex IHC, pairing high abundance markers with lower intensity Alexa Fluor dyes helps prevent signal saturation and minimizes bleed-through between channels after unmixing. Conversely, assigning high intensity dyes to low abundance markers improves their detectability and signal-to-noise ratio, enabling more accurate and balanced visualization across all targets.

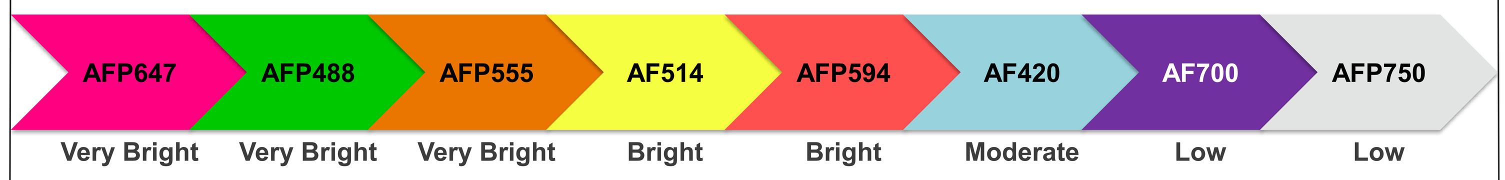

Figure 4. Alexa Fluor dye brightness, in order of high intensity (left) to low intensity (right).

Primary antibody conjugate	Marker intensity healthy colon	Marker intensity colon cancer	Fluor brightness
β-catenin (*15B8*) AF420	++	+++	+
CD68 (*KP1*) AFP488	++	++	+++
CD3 (*RM344*) AF514	++	+	++
CD8a (*C8/144b*) AFP555	++	+	++
P53 (*DO-7*) AFP594	+	+++	+++
PD-1 (*RM309*) AFP647	+	+++	+++
Pan-CK (*AE1/AE3*) AFP700	+++	+++	+
Ki-67 (*SOLA15*) AFP750	+++	+++	+

Table 2. Fluorophore assignment was guided by marker expression levels in both healthy and cancerous colon tissue and Alexa Fluor (AF) brightness, with highly abundant targets paired to lower intensity dyes and lower abundance targets assigned to higher intensity fluorophores to optimize signal balance and detection sensitivity.

2. Balance signal intensities between adjacent channels

Balancing signal intensities across adjacent channels is critical in multiplex IHC to minimize spillover and ensure accurate marker detection when unmixing on spectral imaging platforms. In the initial design, Ki-67 (AF514) exhibited excessive signal that bled into the CD68 (AFP488) channel (Fig. 3A). This was resolved by reassigning fluorophores and balancing two distinct high expression markers into adjacent channels in the final panel (Fig. 3B).

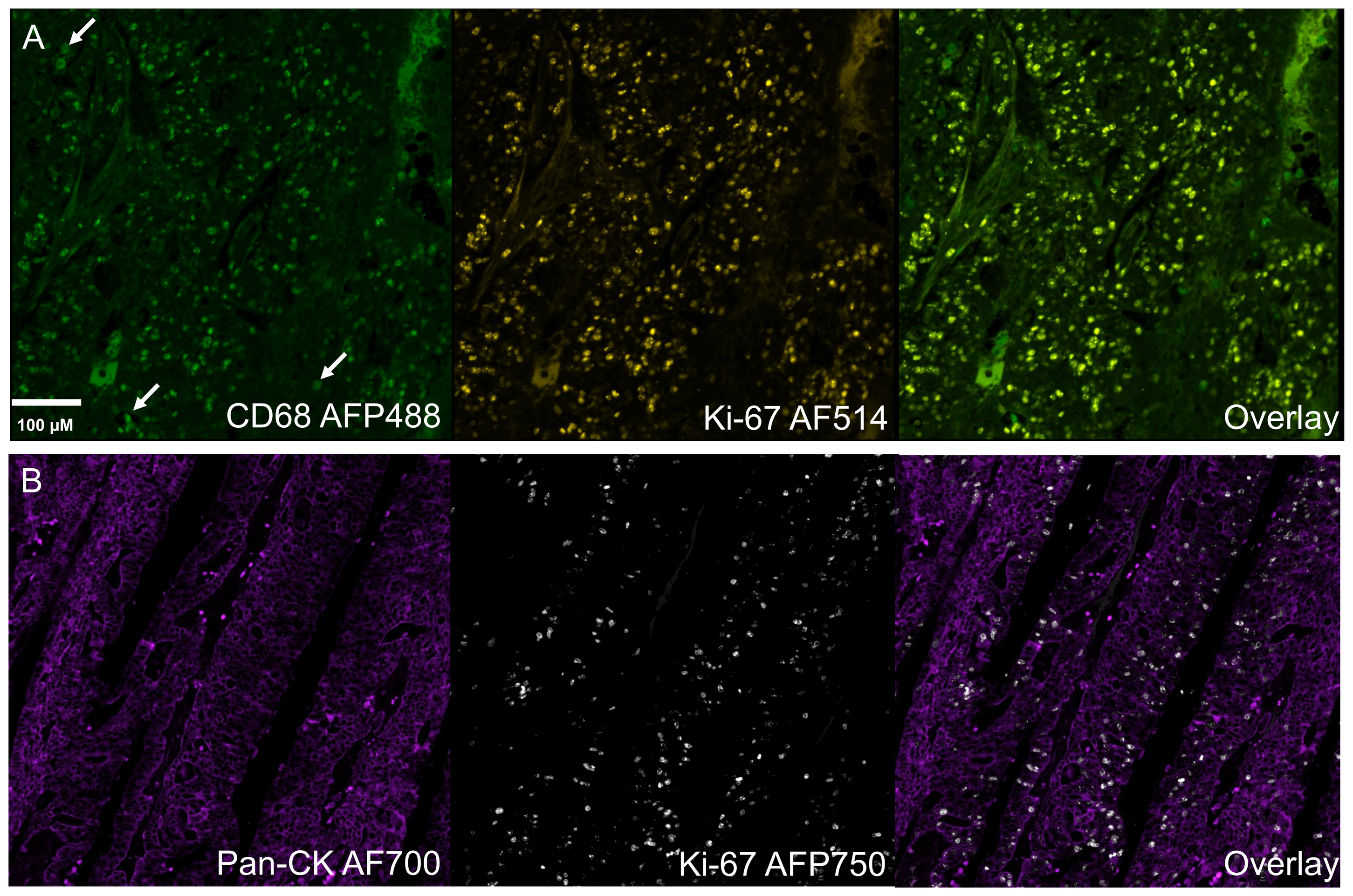

Figure 5. Full multiplex, unmixed 20X images on the EVOS S1000. (A) Ki-67 (AF514) showed substantial bleed-through into the AFP488 channel, obscuring CD68 detection, even at reduced antibody concentration. White arrows denote true CD68 expressing cells. (B) Reassignment of Ki-67 to AFP750 eliminated channel interference and improved signal resolution. Pan-CK was assigned to AF700, with balanced adjacent channel intensities and no detectable bleed-through.

3. Avoid placing colocalized markers in adjacent channels when possible

Spectral overlap between adjacent channels can obscure true co-expression, so marker placement should account for expected colocalization. In this panel, CD68 (AFP488, macrophages) and CD3 (AF514, T cells) were placed in adjacent channels since they are not expected to colocalize, improving channel balance and reducing cross-talk observed in the initial design (**Figure 6C and 6D**). In contrast, co-localizing epithelial markers β-catenin and Pan-CK were assigned to spectrally distant channels (AF420 and AF700) to prevent overlap (**Figure 6C and 6D**).

Methods

All experiments were performed using FFPE healthy or adenocarcinoma colon tissue. As a general guideline, the HIER conditions should be selected to accommodate the most demanding marker in the panel. For this panel, PD-1 requires antigen retrieval for 1 hour in a steamer at pH 9, representing the most stringent condition among the markers. Accordingly, this retrieval method was applied to the entire panel and was found to perform well across all markers.

To reduce high autofluorescence in colon tissue, a white light bleaching step was performed after epitope retrieval and prior to staining using 4.5% H_2O_2 and 24 mM NaOH in 1X PBS with 30 minutes of white light exposure. This step improves staining clarity and brightness in FFPE tissues.

Tissues were stained in a one-step cocktail for 1 hour at room temperature using the concentrations shown in **Table 1**. After washes, DAPI was added as a last step at 1 µg/mL for 5 minutes. Tissues were mounted in ProLong^{TM} Glass Antifade Mountant and allowed to cure overnight before imaging on the EVOS S1000.

Results

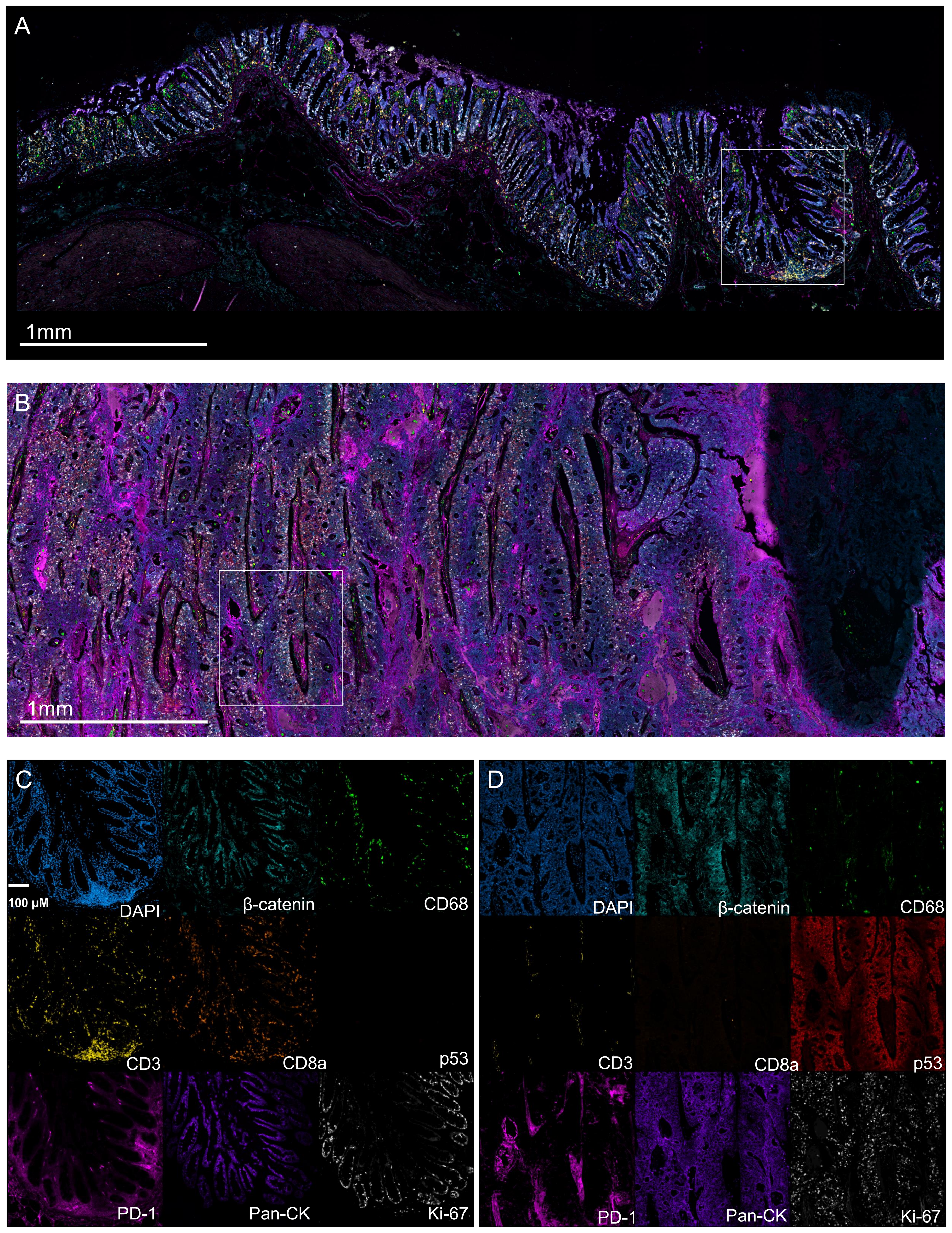

Figure 6. Multiplex IHC distinguishes epithelial structure, proliferation, and immune distribution in colon tissue. Multiplex images acquired on the EVOS S1000 show coordinated detection of epithelial, immune, and proliferation markers in FFPE colon. (A) Healthy tissue displays intact glandular architecture with β-catenin and Pan-CK marking organized epithelial layers. (B) Adenocarcinoma shows disrupted structure and increased cellular density. (C–D) High-magnification views highlight differential marker expression. In healthy tissue (C), Ki-67 is restricted to crypt bases and CD3+/CD8a+ T cells are prevalent, consistent with normal turnover and active immune surveillance. In tumor tissue (D), β-catenin and Pan-CK are expanded and disorganized, Ki-67 and p53 are elevated, and CD3+/CD8a+ T cells are largely absent, indicating dysregulated proliferation and immune exclusion. These patterns demonstrate how multiplex IHC resolves spatial differences in epithelial organization, proliferation, and immune response between healthy and malignant tissue.

Conclusions

A rational, design-driven approach to multiplex IHC—incorporating marker expression levels, fluorophore brightness, channel spacing, and expected co-localization—enabled robust panel performance with minimal spectral spillover and cross-talk. Strategic reassignment of high-abundance markers and optimization of antibody concentrations were critical for achieving balanced signal intensities and preserving data integrity. The final workflow produced high-resolution, reproducible images on the EVOS S1000, with reliable signal separation across eight markers plus DAPI. Biologically relevant differences between healthy colon and adenocarcinoma were clearly resolved, including increased p53 expression, reduced CD3/CD8a T cell infiltration, and disrupted epithelial architecture (Pan-CK, β-catenin) in tumor tissue. Together, this work demonstrates that thoughtful panel design is important for accurate multiplex imaging and enables scalable, high-confidence spatial analysis of complex tissues.

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