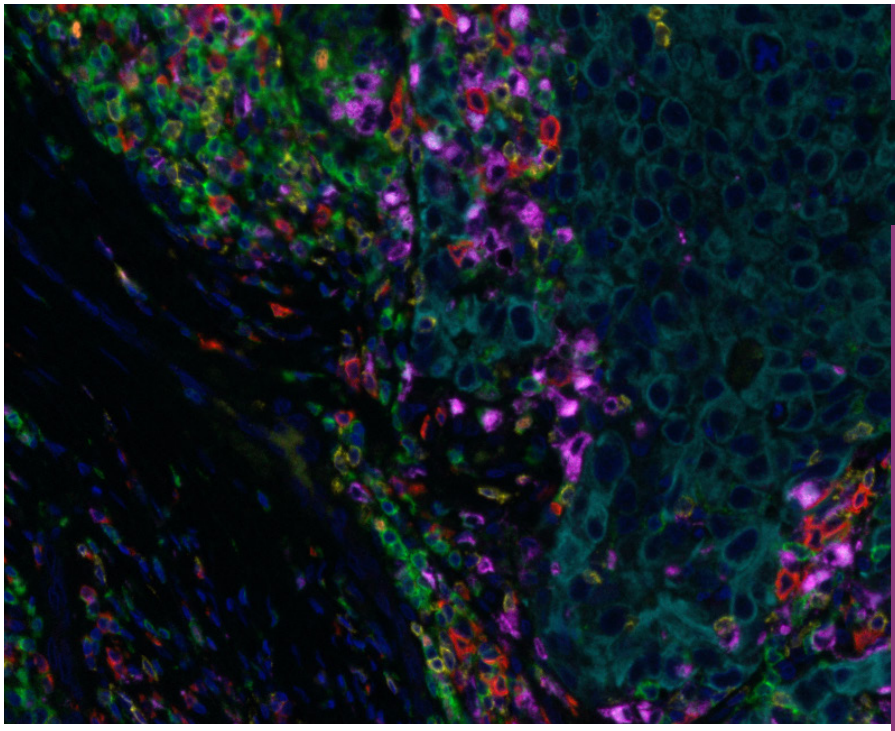

APPLICATION NOTE

Automated Multiplex Biomarker Staining and Imaging

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A Fully Automated Staining Assay Using Fluorescent Multiplex Immunohistochemistry

Introduction

Immuno-oncology research has increasingly leveraged our growing understanding of how cancer suppresses the immune system and how the tumor microenvironment impacts disease progression, for the development of novel therapeutics that re-engage the immune system. Advancing this understanding will involve continued characterization of the interactions that occur among immune cells and cancer cells residing within the tumor and its periphery.

Fluorescent multiplex immunohistochemistry (IHC) assays are uniquely suited to characterizing and quantifying these complex interactions *in situ*. Here we describe a robust, fully-automated 7-color IHC method that significantly shortens the Opal™ procedure time from days to half a day. This automated staining procedure coupled with multispectral imaging for simultaneous detection of up to six tissue biomarkers plus nuclear counterstain, provides the ability to visualize interactions between specific immune and cancer cells within the context of the tumor microenvironment.

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Methods

Automated Opal Staining

Formalin-fixed paraffin-embedded (FFPE) samples of normal tonsil and primary tumors were immunostained using Opal Multiplex Automation IHC Detection Kits (Figure 1) on the BOND RX from Leica Biosystems' BOND RX.

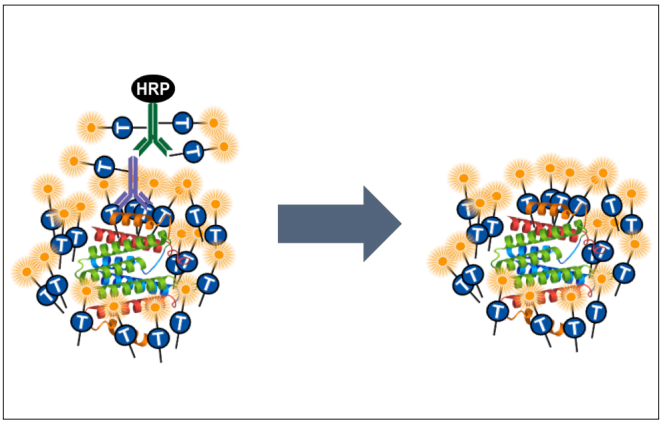

Figure 1. **Opal Detection.** Opal amplification of antigen detection using primary antibody (purple), Opal HRP polymer (green/black) and Opal fluorophores (blue/orange). After deposition of Opal reagents, antibodies are stripped to allow subsequent staining of other antigens.

Sections were baked at 65 °C for three hours then transferred to the BOND RX. All subsequent steps were performed with an automated Opal IHC procedure on the BOND RX (Figure 2). First, sections were subjected to deparaffinization and antigen retrieval. Subsequent Opal staining of each antigen occurred as follows: slides were blocked with PerkinElmer blocking buffer for 10 min then incubated with primary antibodies at optimized concentrations followed by Opal HRP polymer and one of the Opal fluorophores. Individual antibody complexes were stripped after each round of antigen detection. After the final stripping step, DAPI counterstain was applied and slides were removed from the BOND RX for coverslipping. For epitope stability and Opal fluorophore stability tests, each section was only stained with one primary antibody and Opal fluorophore pair, and was subjected to multiple rounds of stripping either before applying the primary antibody (for epitope stability test) or after the detection of Opal fluorophores (for Opal fluorophore stability test).

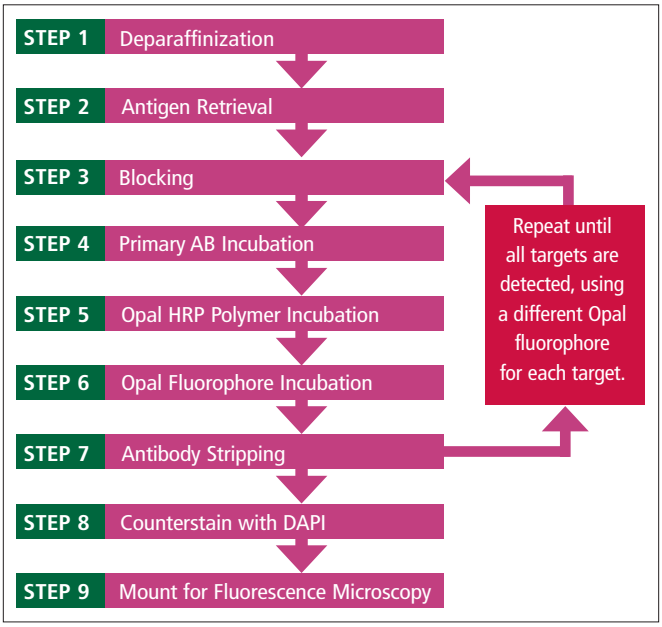

Figure 2. Automated Opal multiplex IHC procedure.

Image Acquisition and Analysis

Stained slides were imaged with a Vectra® 3 Automated Quantitative Pathology Imaging System, and analyzed using inForm® software.

Results

Epitope Stability After Multiple Rounds of Stripping During Automated Opal Staining

Epitope stability during automated staining was assessed on tonsil tissues that were exposed to one, three or six rounds of stripping before staining with Opal 520 using antibodies against either CD4, CD8, CD20 or Ki67 epitopes (Figure 3). Staining intensity was stable within 10-25% after being exposed to up to six stripping steps. Thus, the order of antigen detection can be re-arranged in the automated procedure without substantial changes to epitope availability and resulting signal levels, providing robustness and flexibility for assay development.

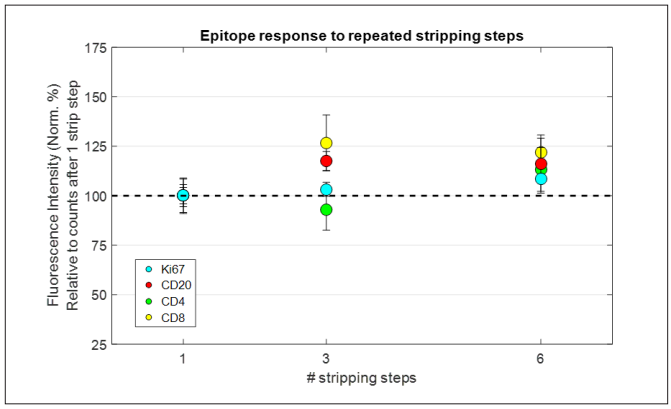

Figure 3. **Tonsil tissue exposed to one, three or six rounds of stripping, followed by Opal 520 staining.** Average staining intensity among three replicate slides was measured using inForm software, then normalized to the average intensity after a single round of stripping. Error bars represent standard deviation.

Opal Fluorophore Stability After Multiple Rounds of Stripping During Automated Opal Staining.

Opal fluorophore stability during automated staining was assessed on tonsil tissues that were exposed to one, three or six rounds of stripping after staining with individual Opal fluorophores using antibodies against CD20 (Opal 520, Opal 540, Opal 570, Opal 620, Opal 650) or PanCK (Opal 690) (Figure 3). All Opal fluorophores were stable to within 25% of the starting intensity after being exposed to up to six stripping steps. Thus, Opal signal degradation does not need to be considered when choosing staining order during protocol development.

Complete Stripping Between Multiplex Staining Steps Using Automated Opal Staining

Efficiency of antibody removal during stripping steps was assessed on tissue stained with a duplex protocol in which primary antibody (CD3, 1:100 dilution) was added in the first step (Opal 520 readout), but not the second step (Opal 570 readout). The Opal 570 readout thus reported on any residual primary antibody remaining from the first step due to insufficient stripping.

An automated procedure with an inefficient stripping protocol showed high levels of residual antibody (Figure 4, left), while the automated Opal staining procedure showed complete antibody stripping (Figure 4, right) that provides confident signal identification in highly multi-plexed IHC assays.

Multiplex Staining of Tumor-infiltrating Lymphocytes (TILs) in Human Breast Cancer Tissue

A fully-automated 7-color IHC protocol was developed using the Opal 7-Color Automation IHC Kit and applied to breast cancer tissue to visualize TILs in the tumor microenvironment (Figure 5). The automated procedure provided exceptional signal isolation allowing detection and isolation of six biomarkers and DAPI counterstain.

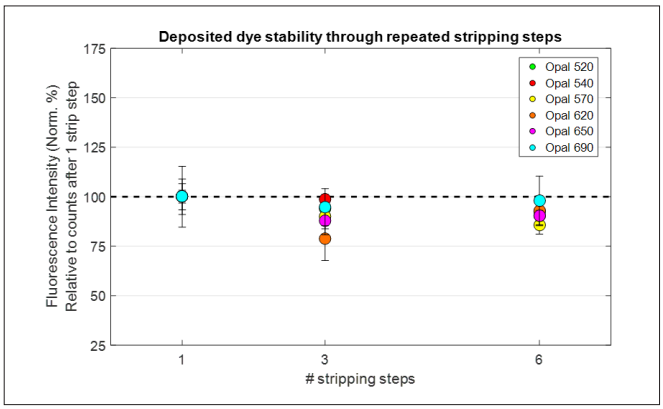

Figure 4 . **Tonsil tissue stained with Opal 520, 540, 570, 620, 650 or 690 followed by one, three or six rounds of stripping.** Average staining intensity from three replicate slides was measured using inForm software, then normalized to the average intensity after a single round of stripping. Error bars represent standard deviation.

Reproducibility Assessment From Staining of Serial Tonsil Sections

To evaluate the reproducibility of the automated Opal staining procedure, 30 serial sections of human tonsil tissue were stained against CD20 with a fully-automated fIHC protocol using Opal 540. The Vectra 3 Automated Quantitative Pathology Imaging System was used to image whole slide scans from which individual fields were marked for multi-spectral imaging (Figure 7, top).

Multi-spectral images were analyzed using inForm software to quantify CD20 staining levels. Reproducibility between the thirty slides was assessed by comparing the average membrane staining intensity of all cells within each image (Figure 7, bottom). Variability was within 12% CV.

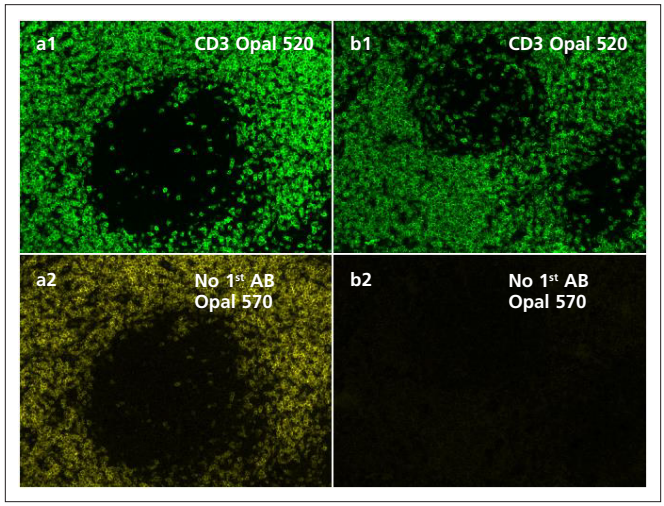

Figure 5. **Tonsil tissue sections were stained with CD3-Opal 520 followed by antibody stripping, then labeled with Opal HRP polymer and Opal 570 to detect unstripped antibody. (a1,b1)** CD3-Opal 520 signal. **(a2)** Opal 570 signal from the same slide as **(a1)** demonstrating incomplete stripping with an inefficient stripping method; **(b2)** Opal 570 signal from the same slide as **(b1)** showing complete stripping of antibodies using the automated Opal procedure. Images of the same Opal dye are shown at the same contrast.

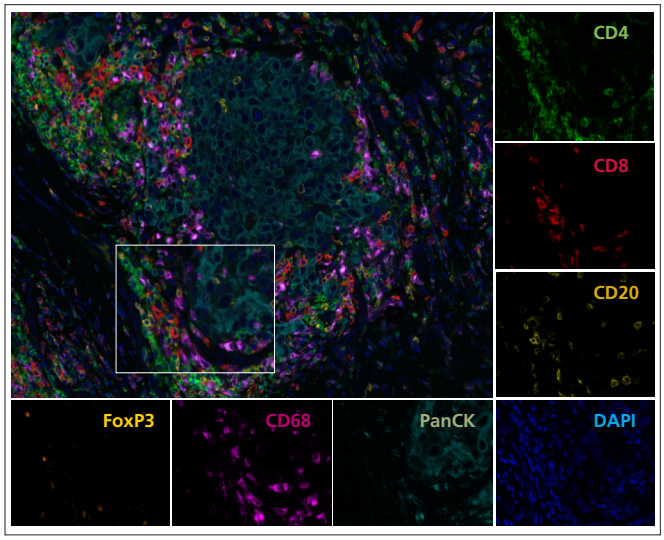

Figure 6. **Human breast cancer tissue was stained against CD4, CD8, CD20, FoxP3, CD68 and PanCK using the Opal 7-Color Automation IHC Kit on the BOND RX automated IHC & ISH stainer.** Images were captured using the Vectra 3 Automated Quantitative Pathology Imaging System and image analysis was performed using inForm software.

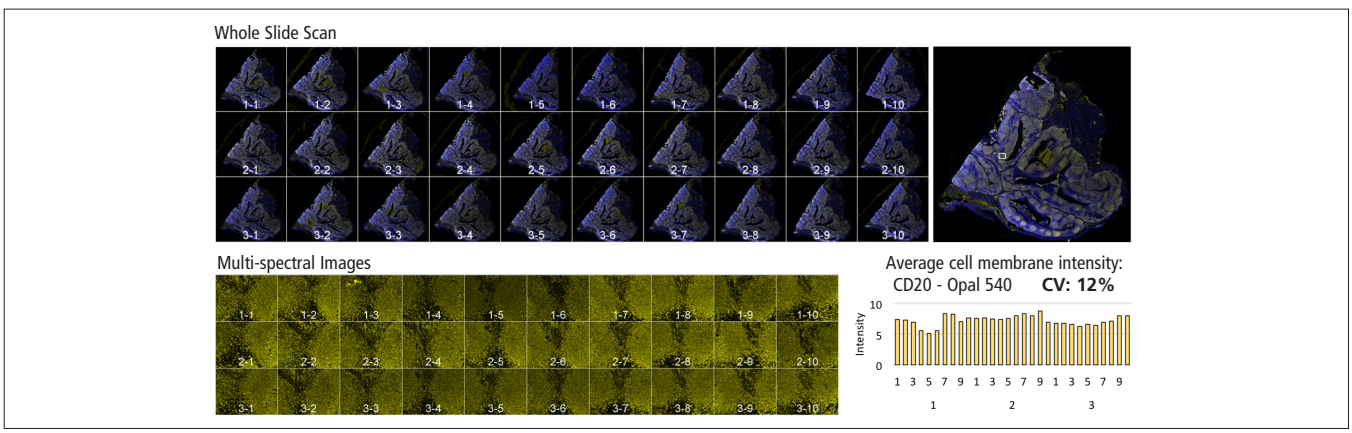

Figure 7 . **Staining reproducibility of serial tissue sections.** Top Left: Whole slide scan images of tonsil tissue stained with Opal 540 against CD20 using the Automated Opal staining protocol on the BOND RX. Labels indicate slide position with in the BOND RX (Tray-Position). Bottom left: Multispectral images (MSIs) from each tissue shown on the top left. **Top** Right: Representative image showing the approximate location of the MSIs. Bottom Right: CD20 staining intensity quantified from the MSIs. Staining variability was within 12% CV.

Conclusion

We developed a novel fully-automated Opal 7-color IHC staining assay for the BOND RX. The described approach transforms the traditional multi-day protocol to a high throughput solution that can be run overnight. We observed minimal degradation of fluorescence signal and epitope availability during automated staining, complete inactivation between staining steps, and minimal crosstalk in multiplex staining of breast cancer tissue. This fully-automated staining assay is a robust method to increase the throughput of multiplex tissue staining while substantially reducing hands-on time.

For more information, please visit www.perkinelmer.com/Phenoptics

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