Automated Opal multiplex immunofluorescence and PhenoCode Signature Panel staining on the Parhelia Spatial Station

enables reproducible, high-quality spatial phenotyping of the tumor microenvironment in FFPE tissues

Abstract

Multiplex immunofluorescence (mIF) using Quanterix (formerly Akoya) Biosciences Opal tyramide signal amplification (TSA) chemistry and PhenoCode Signature Panels (PSP) provides detailed spatial characterization of the tumor microenvironment (TME) in formalin-fixed paraffin-embedded (FFPE) tissues. However, manual execution of these multi-step staining protocols is labor-intensive, time-consuming, and susceptible to inter-operator variability. Here, we demonstrate the successful automation of both custom Opal mIF panels and pre-designed PhenoCode Signature Panels on the Parhelia Spatial Station. Using human lung cancer and tonsil FFPE specimens, we show that automated staining produces high-quality, spectrally resolved, multiplex fluorescence images with distinct signal for each Opal channel. The results demonstrate clear visualization of immune and tumor markers across 7–8-plex panels, validating the Parhelia Spatial Station as a robust platform for automated Opal and PSP workflows that improves reproducibility while reducing hands-on time.

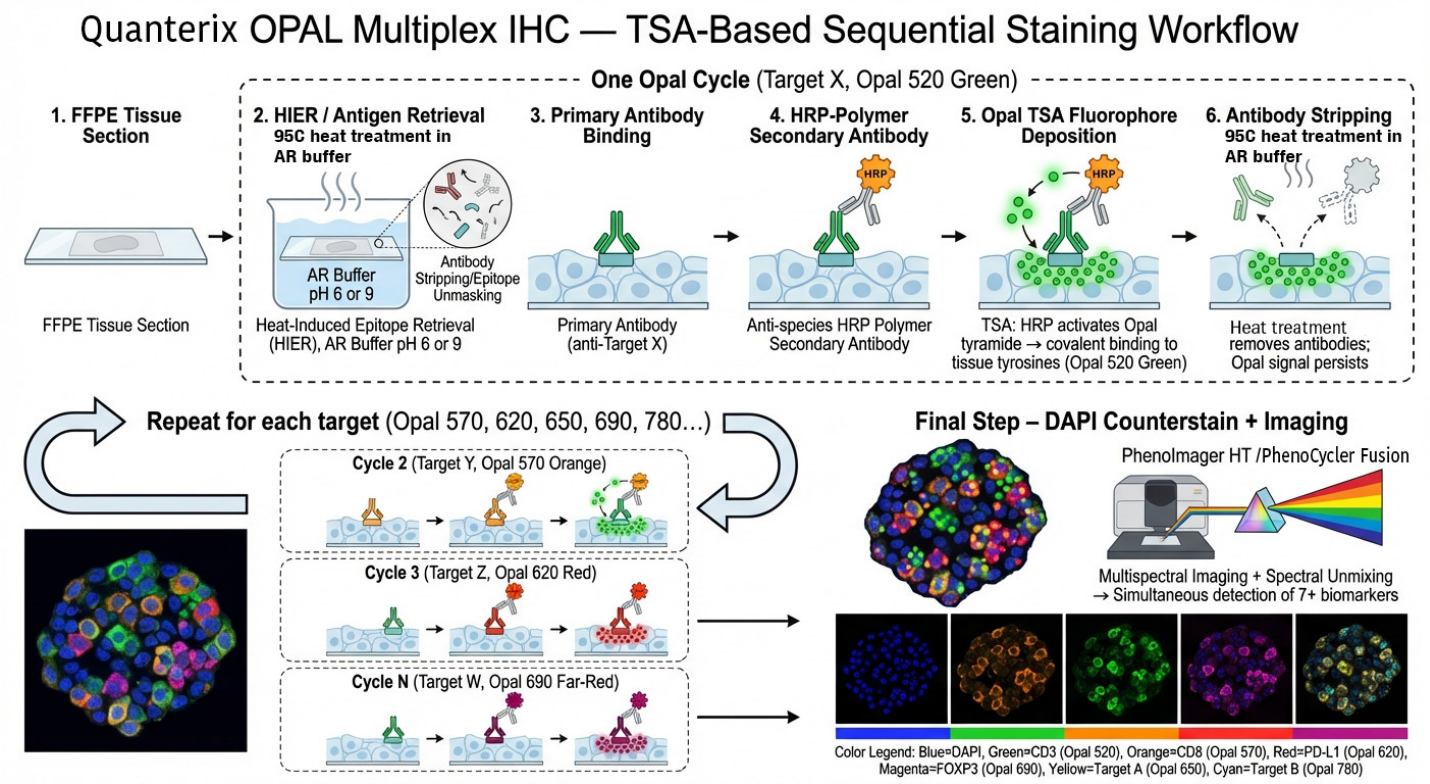

Figure 1. Schematic diagram of Quanterix (formerly Akoya) OPAL staining technology.

Introduction

Spatial biology has rapidly advanced our ability to characterize the cellular composition and architecture of the tumor microenvironment (TME). Multiplex immunofluorescence (mIF) using tyramide signal amplification (TSA) enables the simultaneous detection of multiple biomarkers within a single FFPE tissue section, preserving crucial spatial context that is lost with techniques requiring tissue dissociation.

Quanterix (formerly Akoya) Biosciences' Opal TSA chemistry is a well-established platform for mIF, supporting the covalent deposition of spectrally distinct fluorophores at protein target sites (**Figure 1**). This approach enables the construction of multiplex panels of up to 8+ markers (including DAPI nuclear counterstain) while maintaining excellent signal-to-noise ratios and minimal spectral crosstalk. The more recent PhenoCode Signature Panels (PSP) combine barcode-based antibody chemistry from the PhenoCycler platform with Opal TSA signal amplification, providing pre-validated 6-plex kits with one customizable open channel.

Despite these technological advances, manual execution of Opal and PSP staining protocols remains a significant bottleneck. The dewaxing, antigen retrieval followed by iterative cycles of blocking, primary antibody incubation, secondary antibody incubation Opal dye deposition and primary antibody complex stripping (done by microwave or heat treatment) are both time-consuming (often exceeding 12 hours of active hands-on time for a single panel) and prone to variability between operators and staining sessions.

The Parhelia Spatial Station is a purpose-built, open-chemistry robotic platform designed to automate multi-step spatial biology sample preparation workflows. Its capillary gap staining technology ensures uniform reagent coverage while minimizing consumption, and its precision temperature control (0–100˚C) supports onboard dewaxing, heat-induced epitope retrieval (HIER), heat driven antibody stripping and temperature-sensitive incubation steps. Here, we report the application of the Parhelia Spatial Station to automate both custom Opal mIF panels and PhenoCode Signature Panel staining across multiple tissue types.

Materials and Methods

Tissue specimens

Human lung cancer FFPE sections were provided by the City of Hope Comprehensive Cancer Center (COH) and Wayne State University. Human tonsil FFPE sections were prepared at Wayne State University. All tissues were sectioned at 4 µm and mounted on positively charged glass slides.

Custom Opal panel design

Two custom Opal panels were designed and optimized. Panel 1 comprising 6 markers: CD8 (Opal 480), CD20 (Opal 520), CD27 (Opal 570), Cytokeratin (CK, Opal 620), CD3 (Opal 690) and DAPI for nuclear counterstaining. Panel 2 comprising a 7-plex configuration targeting: usp22 (Opal 480), CD86 (Opal 520), usp7 (Opal 570), CD206 (Opal 620), aquaporin (Opal 690), and sftpc (Opal 780), plus DAPI nuclear counterstain. Antibody-Opal pairing was guided by target abundance and fluorophore brightness.

PhenoCode Signature Panel

The pan-immune PhenoCode Signature Panel was configured as a modified PSP 6-plex on tonsil tissue with the following targets: CD8 (Opal 480), Ki67 (Opal 520), PD-1 (Opal 570), CD20 (Opal 620), Foxp3 (Opal 690), and CD4 via TSA-DIG amplification with Opal 780 readout. Antibody dilutions were optimized per the manufacturer's recommendations (see Table 2).

Automated staining on the Parhelia Spatial Station

All staining was performed on the Parhelia Spatial Station using the ST12 Slide Staining Module with capillary gap exchange. Protocols were designed and exported via Parhelia StainWorks software. The automated workflow included: (1) onboard dewaxing using Parhelia Dewax reagent; (2) heat-induced epitope retrieval (HIER) at 95˚C in AR6 or AR9 buffer; (3) sequential cycles of blocking, primary antibody incubation, HRP-conjugated secondary antibody incubation, and Opal fluorophore deposition; (4) microwave-assisted antibody stripping between cycles; and (5) DAPI nuclear counterstaining and coverslipping.

Imaging

Stained slides were imaged on an Quanterix (formerly – Akoya) PhenoImager system. Whole-slide multispectral images were acquired and spectrally unmixed to resolve individual Opal channels. Composite images were generated for visualization of marker co-expression and spatial distribution patterns

Results

Parhelia ST12 Slide Staining Module: Precision Thermal Engineering for Multiplexed TSA-IF

Key to the performance of the Parhelia Spatial station is the Parhelia ST12 Slide Staining Module (**Figure 2**) — an engineering solution purpose-built for the demands of complex, multistep staining protocols. The module uniquely combines prolonged low-temperature incubations, which promote the uniform distribution of staining reagents and eliminate concentration gradients across the tissue section, with repeated high-temperature stripping cycles reaching up to 98°C. This broad thermal range is made possible by a fully sealed, internally humidity-controlled chamber that maintains optimal moisture throughout the run, paired with precision-engineered slide support shelves that ensure homogeneous heat distribution across every slide position. Together, these design principles address the critical technical requirements of OPAL multiplexed immunofluorescence — making the Parhelia the ideal platform for the reliable, reproducible, and automated execution of Opal staining workflows on the Spatial Station.

Addressing TSA Uniformity Challenges in Capillary Gap Format

Automating Opal assay in capillary gap format is known to run into uniformity issues, which happen due to rapid consumption of the TSA reagents as the solution flows over the tissue, leading to gradient artifacts, whereby the lower portion of the tissue shows less staining intensity. This effect is especially obvious with several tissues placed one under another on the same slide. We developed significant optimizations to remedy this effect. First, we leverage Parhelia Spatial Station's unique capabilities of cooling tissue samples below room temperature, significantly slowing down HRP activity and thus letting the TSA cocktail distribute more evenly before the dye gets depleted by the peroxidase reaction. We found that this cooling already leads to improved uniformity compared to doing TSA at RT (data not shown). In addition, we tested whether applying two aliquots of the TSA reagent mixture (100µl + 100µl) interspersed by 10 minute incubation at 4°C would produce better uniformity and indeed, in a side-by-side comparison, we found that performing the so-called "double add" is sufficient to produce uniform staining over two large (10×2 mm) serial sections of mouse spleen stacked one above the other on the same slide **(Figure 4).**

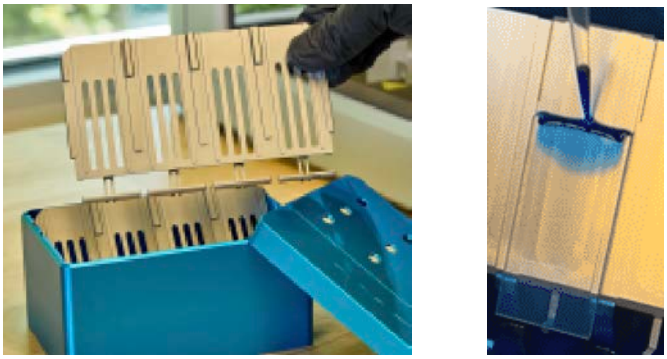

Figure 2. Left: Parhelia ST12 Slide Staining Module. **Right:** demonstration of capillary gap liquid exchange, which is at the core of Parhelia staining automation.

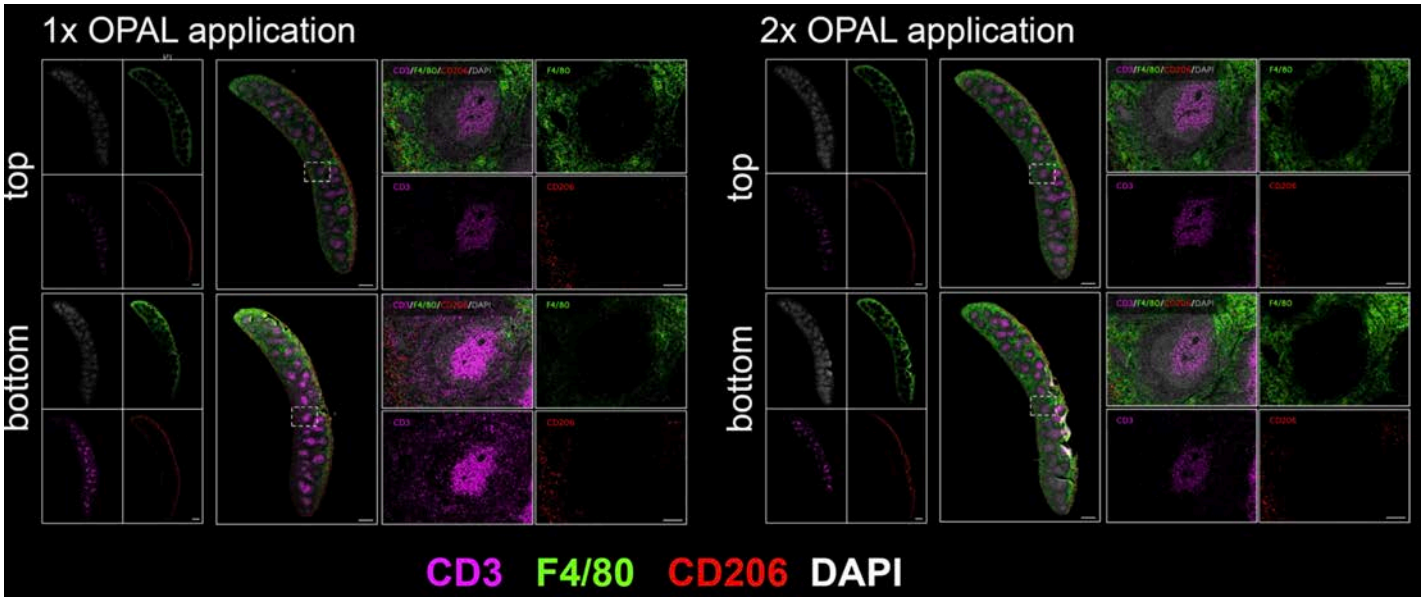

Figure 3. Uniformity optimization for TSA in capillary gap staining. Side-by-side comparison of 1X (left) versus 2X ("double add") Opal TSA application on serial sections of mouse spleen stacked vertically on the same slide. Top and bottom tissue positions are shown for each condition. Scale bar: 0.5 mm (overview), 100 µm (insets). Images and data courtesy of Julia Zimmerman and Özlem Akili, TRON Mainz.

Opal mIF on human lung cancer (City of Hope)

At first, a 6-plex Opal panel was validated on a large section of human lung cancer FFPE tissue (approximately 13x20mm). The data obtained in this test confirmed high signal-to-noise ratio, specificity and uniformity across a variety of structural and immune markers **(Figure 4)**. At that point, the team was comfortable moving forward with a more complex complex 7-plex panel capturing a broader variety of immune and tumor markers relevant to lung cancer biology, with the last channel stained via TSA-DIG deposition followed by an anti-DIG Opal 780 antibody.

The 7-plex Opal staining produced a richly detailed composite image revealing the heterogeneous spatial distribution of immune and tumor-associated markers (**Figure 5**). All seven Opal channels demonstrated strong, specific signal with clear spectral separation. The composite image reveals distinct tissue compartments: regions of sftpc+ epithelial tumor cells (Opal 780, magenta), interspersed with CD86+ myeloid cells (Opal 520, green) and CD206+ macrophages (Opal 620). DAPI nuclear staining provided clear delineation of individual cells throughout the entire section.

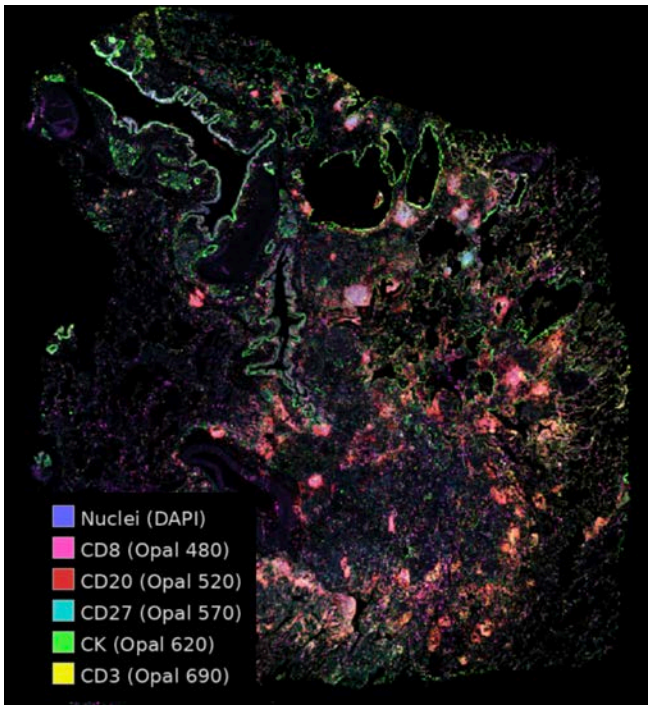

Figure 4. A very large section of human lung cancer (13x20mm) stained using optimized double-add TSA conditions on the Parhelia Spatial Station. Data courtesy of Britney Oeung and Colt Egelston, City of Hope

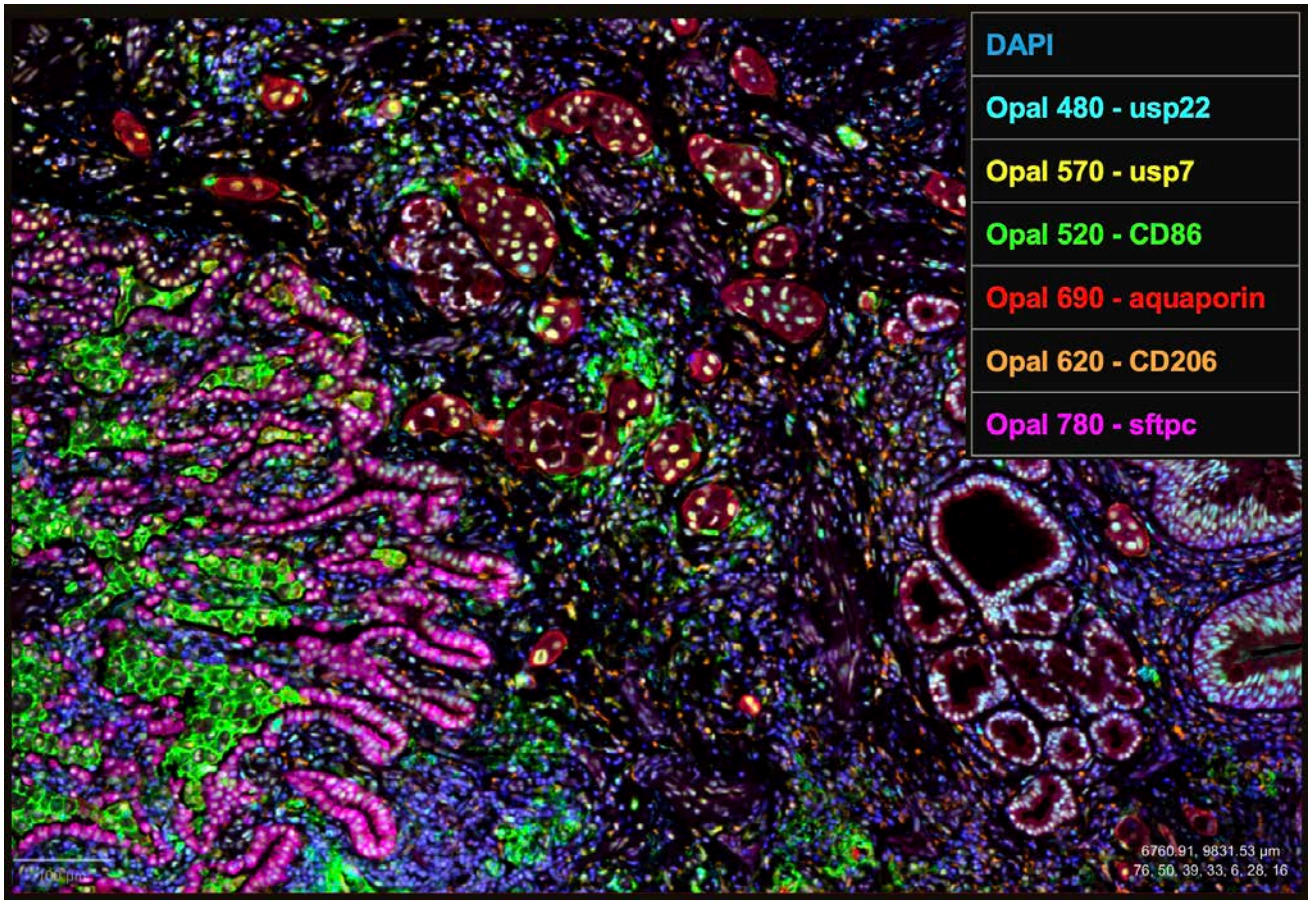

Figure 5. Automated 7-plex Opal mIF staining of human lung cancer FFPE tissue on the Parhelia Spatial Station. Scale bar: 100 µm. Data courtesy of Britney Oeung and Colt Egelston, City of Hope Comprehensive Cancer Center.

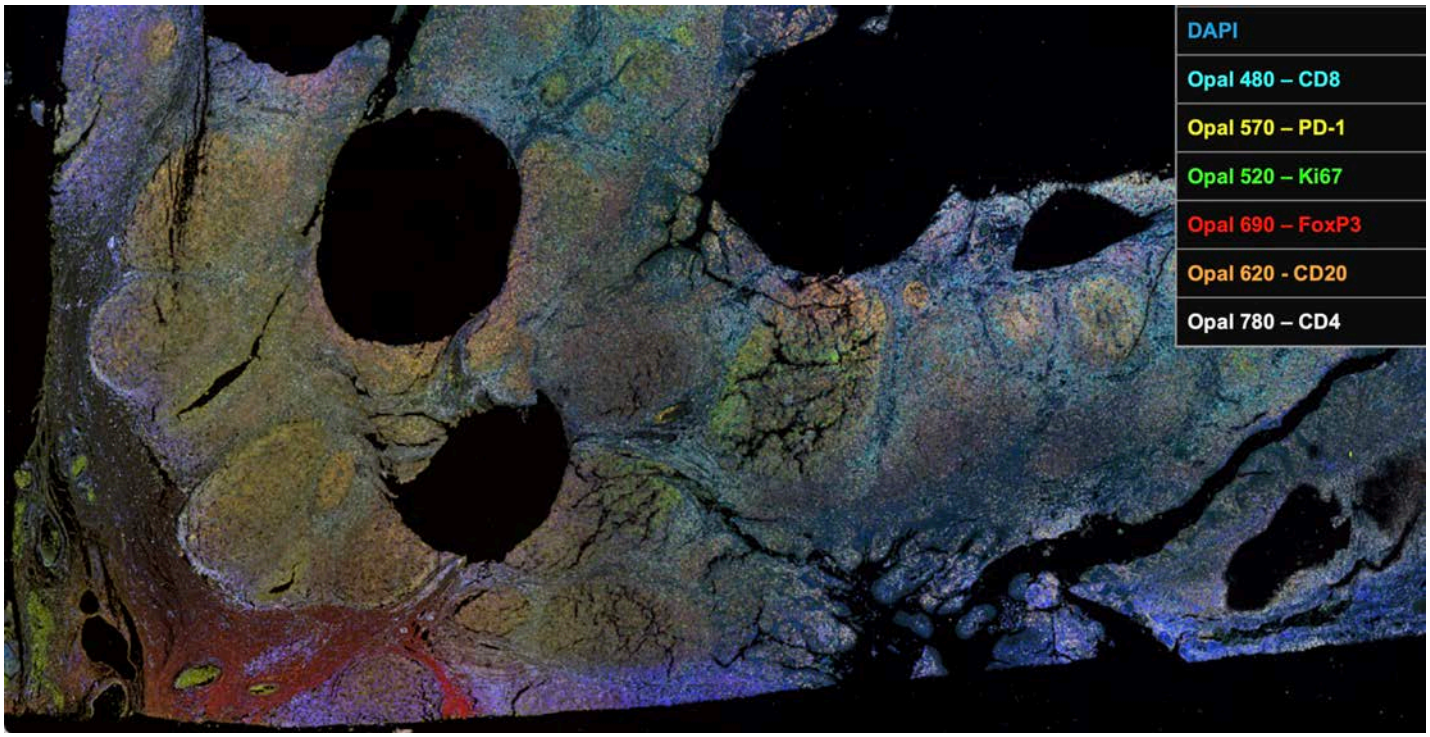

Figure 6. Automated PhenoCode Signature Panel staining of human tonsil FFPE tissue on the Parhelia Spatial Station. Whole-section multispectral composite showing CD8 (Opal 480, blue), Ki67 (Opal 520, green), PD-1 (Opal 570, yellow), CD20 (Opal 620, orange), Foxp3 (Opal 690, red), CD4 (Opal 780, white), DAPI (blue), and autofluorescence (AF, gray). Scale bar: 600 µm. Data courtesy of Jessica Back, PhD and George Schwenkel, Wayne State University.

PhenoCode Signature Panel on human tonsil

The PhenoCode Signature (PSP) method is different from the regular Opal. It uses primary antibodies conjugated to DNA tags, a reporter oligo with HRP and regular Opal dye, followed by a mild stripping treatment as opposed to high heat. In collaboration with a flow core at Wayne State (Jessica Back), we modified the previously developed 7-color Opal protocol, adjusting buffers, steps, temperatures and incubation times in accordance with Quanterix's (formerly Akoya's) standard PhenoCode Signature protocol.

Thus automated PhenoCode Signature Panel staining of human tonsil tissue produced a whole-section overview demonstrating uniform staining across the entire specimen (**Figure 6**). The tonsil provides an ideal validation tissue for immune panels due to its well-characterized lymphoid architecture. The composite image reveals the expected spatial organization of immune cell populations: CD20+ B cell follicles, interfollicular T cell zones enriched for CD4+ and CD8+ cells, and scattered Ki67+ proliferating cells concentrated in germinal centers. PD-1 expression is localized to follicular regions consistent with T follicular helper cell distribution, and Foxp3+ regulatory T cells are distributed throughout the T cell zones.

Discussion

The results presented here demonstrate that the Parhelia Spatial Station is capable of fully automating both custom Opal multiplex immunofluorescence panels and PhenoCode Signature Panel staining protocols on FFPE tissues. Across all panel configurations and tissue types tested, automated staining produced high-quality multispectral images with strong, specific fluorescent signals and clean spectral separation between channels.

The successful automation of 6–7-plex Opal panels is particularly noteworthy given the complexity of the sequential staining workflow. Each marker requires a complete cycle of blocking, primary antibody incubation, HRP-polymer incubation, Opal dye deposition, and heat-mediated antibody stripping. Manual execution of these steps introduces cumulative variability, especially in temperature control during HIER and stripping steps, incubation timing, and reagent volumes. The Parhelia Spatial Station's capillary gap staining technology ensures uniform reagent coverage with minimal consumption, while its programmable temperature module maintains precise thermal conditions throughout the protocol.

The PhenoCode Signature Panel results on tonsil tissue validate that the barcode-based antibody cocktail approach, which allows all primary antibodies to be applied simultaneously before sequential detection, is fully compatible with automated workflows on the Spatial Station. This is significant because PSP chemistry represents a distinct workflow from

traditional sequential Opal staining, requiring different handling of the initial cocktail incubation and subsequent barcode-directed detection steps.

The ability to automate these protocols offers several practical advantages for research laboratories: reduced hands-on time (from approximately 8+ hours to walkaway automation), improved batch-to-batch consistency, precise reagent management through the StainWorks software interface, and the ability to process multiple slides in parallel using the ST12 module. These benefits are especially impactful for large-scale translational studies where staining reproducibility across many patient samples is critical.

Conclusions

We have demonstrated that the Parhelia Spatial Station provides a robust, automated platform for Opal multiplex immunofluorescence and PhenoCode Signature Panel staining of FFPE tissues. Custom 7-plex Opal panels on human lung cancer and a modified 6-plex PSP on human tonsil all produced high-quality multispectral images with clear marker-specific signals. Automation on the Spatial Station substantially reduces manual labor, minimizes operator-dependent variability, and supports standardized workflows suitable for both discovery research and translational studies interrogating the tumor microenvironment.

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