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Validation of an automated PhenoCycler-Fusion slide staining protocol on the Parhelia Spatial Station™

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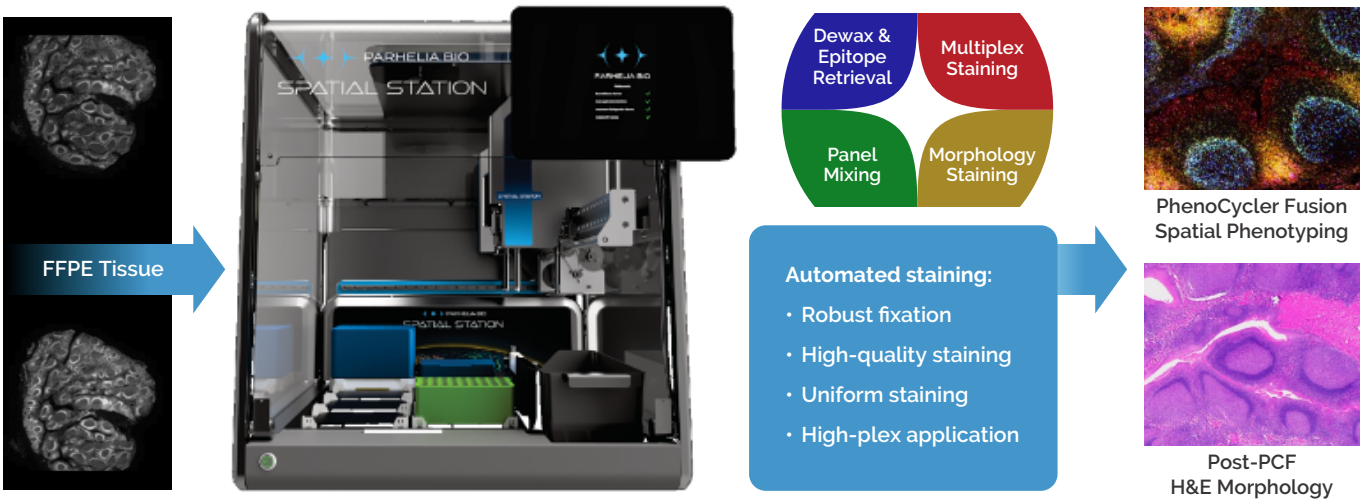
Abstract

Spatial biology encompasses a dynamic and growing menu of assays for interrogating the tissue microenvironment at single-cell resolution. Despite these techniques' increasing multiplexing and multiomic capabilities, the multistep sample processing and staining workflows are mainly accomplished manually. In this report, we describe the automation of the staining protocol for PhenoCycler Fusion (PCF), a popular spatial biology proteomics assay, using the Parhelia Spatial Station. Robust fixation and equivalent staining of two tonsil specimens on the same slide demonstrate the reliability and staining uniformity of the automated method, respectively. Parallel manual and automated multiplexed staining of tonsil samples yield equivalent staining quality. Automated staining of a high-plex panel reveals high-quality spatial topology of tonsillar structure, lymphocyte, and myeloid cell contexture. Finally, we automated the hematoxylin and eosin (H&E) staining protocol after PCF imaging, permitting multiplexed imaging and morphology visualization on the same specimen. Overall, these results validate the automation of the PCF staining workflow, enabling assay standardization for immunological and other discovery and translational spatial biology applications.



Graphical abstract overview of the PhenoCycler Fusion assay sample prep automation on the Parhelia Spatial Station.

Standardizing the spatial biology workflow

Spatial biology is a rapidly growing field that utilizes different technologies to explore the genomic, transcriptomic, proteomic, and metabolomic organization and interactome within the tissue microenvironment at single-cell resolution.[1] These techniques share common sample preparation characteristics, including mounting thin specimens on slides or coverslips, tissue preservation (e.g., formalin-plus-paraffin or fresh-frozen) and its subsequent removal, and application of chemical or biological probes (i.e., staining).

Staining protocols universally comprise multiple manual steps, which are inherently error-prone, demand the attention and time of skilled researchers, and invite intra- and inter-operator inconsistencies and variabilities. Parhelia Biosciences is addressing the challenges of manual processing with dedicated automation solutions that address gaps in laboratory efficiency and standardize the sample preparation workflow for any spatial biology assay.

PhenoCycler Fusion (formally CODEX) assay

The PhenoCycler technology is a leading spatial proteomics technology capable of detecting more than 100 targets in whole tissues at single-cell resolution.[2,3] The assay is designed to run in the PhenoCycler-Fusion (PCF) system, a benchtop spatial biology imager. Briefly, PhenoCycler antibodies corresponding to cellular targets are labeled with unique oligonucleotide barcodes. PhenoCycler reporters comprising the complimentary oligonucleotide sequence tagged with a fluorescent dye undergo hybridization with the antibody-barcode conjugate. For multiplexing, the PCF system fully automates iterative cycles of revealing, imaging, and removing fluorescent reporters (see Graphical Abstract) in diverse tissue types: [4,5]

1. Three fluorescent reporters are revealed to their corresponding barcode-conjugated antibodies.
2. The barcode-conjugated antibodies and reporters are imaged via fluorescent optics.
3. The three reporters are removed, and the cycle repeats for the sequential three reporters.

While the PCF system automates the cyclic imaging process, researchers must manually stain the slides with antibodies and prepare a "reporter plate" consisting of the triplicate fluorescent reporter probes. For formalin-fixed paraffin-embedded (FFPE) samples, the workflow also includes baking, dewaxing, and epitope-retrieval processing. These mundane yet critically important processing and staining procedures include upwards of 60 timely and attention-demanding steps accomplished over 1–3 days, requiring additional planning on behalf of the research team.[6] Furthermore, intra- and inter-operator variability play outsized roles in such multistep procedures, introducing error and risking the repeating experiments with precious, sometimes irreplaceable patient samples (**Table 1**).

Table 1. Summary of challenges with the PCF assay

Challenge	PhenoCycler Fusion
Manual pipetting and hands-on-time	Many short but critical staining, fixing, and washing steps require largely undivided attention of the experimenter Intra and inter-operator variability associated with dozens of manual preparation steps
Reliable and efficient fixation	Multiple washes, antibody applications, and imaging necessitate robust fixation, the loss of which results in rapidly degraded signal
Variability in staining	Non-uniform staining can arise from staining reagent gradients across a slide

Parhelia Spatial Station™: purpose-built spatial biology sample preparation

To circumvent the manual processing and staining procedure associated with the PCF assay, we automated the workflow on the Parhelia Spatial Station.

The Parhelia Spatial Station encompasses state-of-the-art liquid-handling robotics, temperature control, and quality control controlled through a touchscreen user interface. System software includes verified methods, such as the method described here for PCF, and the ability to create, customize, and optimize assays per user (**Figure 1**).

Reagent and probe application, incubation, and buffer exchange are accomplished through patented Capillary Laminar Flow Technology, whereby a ~70 µm capillary gap creates a reaction chamber filled with ~100 µl of liquid. The resulting flow cell also protects specimens from damage and dehydration, circumventing the need for parafilm application during long incubations. Additionally, researchers can achieve significant reagent savings due to the miniaturization of the assay.

Automated slide processing, fixation, and staining for PCF imaging

The manual processing and staining protocol for preparing FFPE samples for PCF imaging was recently published, and the methodology described here reflects that procedure unless otherwise noted.[4] The method utilizes Copley jars for buffer incubations and washes and the application of parafilm coverings during the long antibody staining step. Each step requires the user's full attention and careful execution; noncompliance or timing differences can impact the reproducibility of the study.

Automated staining on the PSS involves assembling a capillary reaction chamber by applying a removable Parhelia CoverPad to each slide. Up to 12 flow cells slide assemblies can be loaded into an S12 slide chamber and placed into the PSS with other reagents. The CoverPads are removed before PCF Flowcell assembly and imaging on the PCF instrument.

To compare the performance of manual and automated PhenoCycler protocols, commercially available FFPE tonsil slides were used (Zyagen, San Diego, CA; two sections on each). FFPE samples manually underwent baking, paraffin dewaxing, tissue rehydration, and heat-induced epitope retrieval (Tris-EDTA pH 9, Agilent Dako), as described in the published STAR method.[4] Furthermore, the reporter plate was prepared manually. While these steps can be completely automated on the PSS, we sought to focus on the PCF staining specifically for this report and compare manual vs automated methods only for the staining workflow.

Figure 2 summarizes the staining procedure. The steps in blue boxes were executed manually (Manual) or automatically on the PSS (PSS-Auto). PSS procedures are built from the touchscreen, and the verified method described here is preloaded on the instrument. The user can customize the protocol to accommodate different incubation times and temperatures. For each step, 150 µl

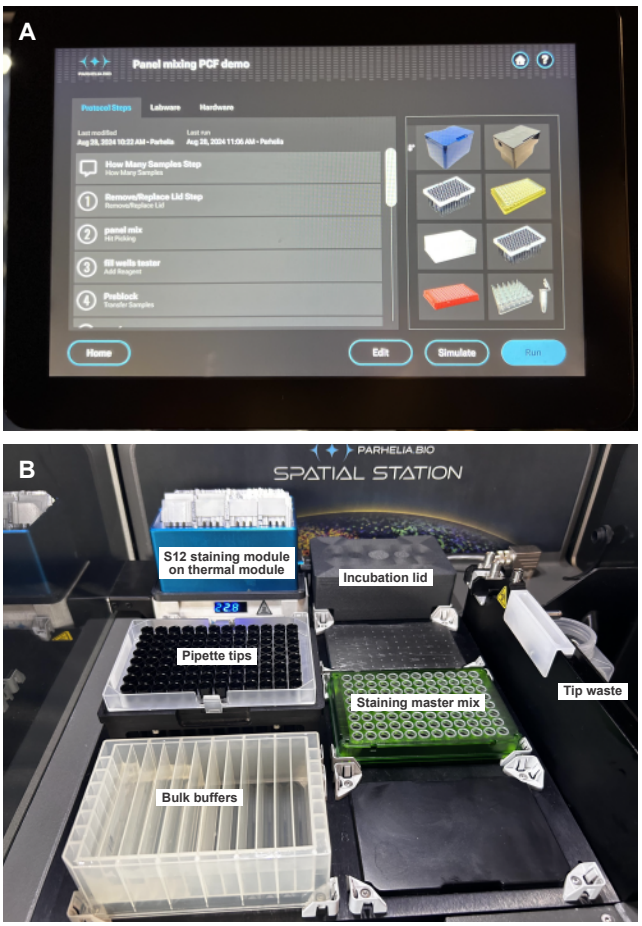


Figure 1. PCF assay setup on the PSS. (A) Touchscreen interface to customize and start runs, (B) typical component arrangement on the instrument deck.

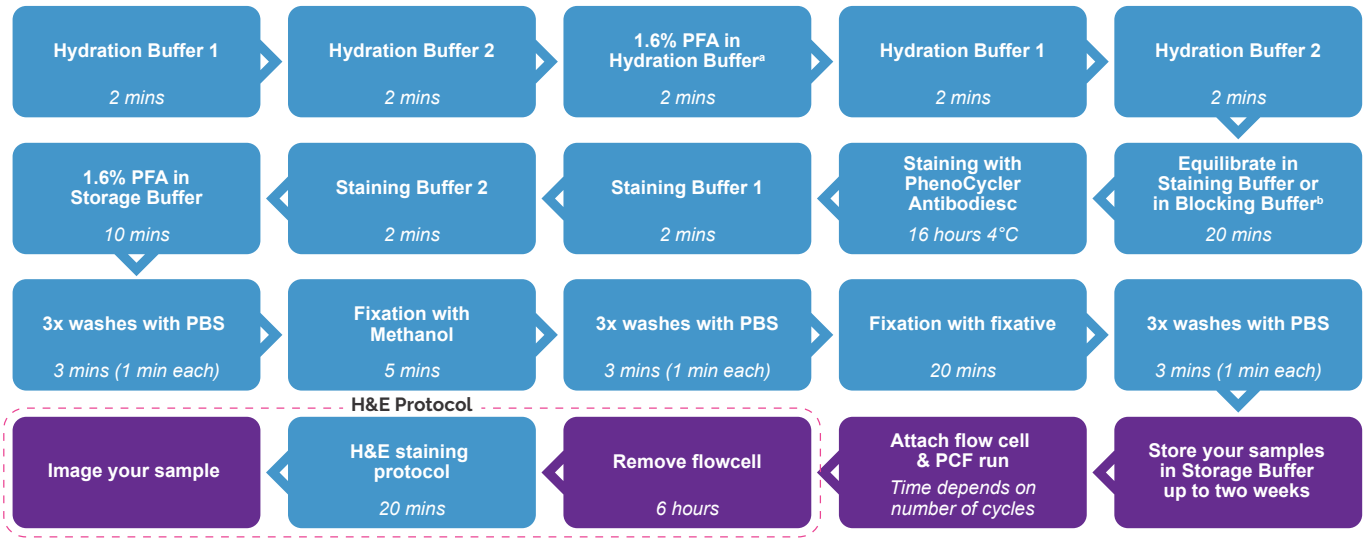


Figure 2. Schematic of the PCF staining procedure and post-PCF imaging H&E staining. Steps automated on the PSS are colored blue, while steps occurring outside the instrument are colored purple. aThis step can be skipped for FFPE tissue samples. bIn this step, blocking buffer (N, S, and J blockers in staining buffer) can be used for the pre-blocking step instead of staining buffer. cAntibody incubation time may vary (e.g., the published method calls for overnight incubation at 4oC, yet FF and FFPE can be incubated at RT for 2 or 3 hours, respectively).

of the specified reagent is robotically delivered to the flowcell. Purple steps were executed outside the PSS, and an optional, post-PCF imaging hematoxylin and eosin (H&E) stain is highlighted in pink.

Manual and PSS-Auto procedures were performed in parallel using the same 15-plex antibody cocktail, reagents, and PCF instrument (**Supplementary Table 1**). A 27-plex antibody panel stained exclusively with the PSS was also imaged to demonstrate staining uniformity and high-plex capabilities (**Supplementary Table 2**). PhenoCycler flow cells were applied, and samples were imaged within 24 hours of staining to avoid any antibody deterioration tissue degradation unless otherwise noted.

Upon completion of PCF imaging, the PCF FlowCells were removed by incubation in xylene for 6 hours. A standard H&E staining protocol was performed on the PSS to automate the evaluation of tissue morphology and integrity.

Results

Uncompromised fixation demonstrates method adoption reliability

The PhenoCycler Assay comprises three fixation steps after antibody staining. In high-plex panels that require multiple wash cycles, robust fixation ensures the antibodies remain bound to tissue sites. Automated assay adoption should demonstrate no loss in antibody signal after sequential imaging cycles. The following represent deviations from the published fixation method performed manually in translating to PSS automation:

1. Room temperature methanol was used for PSS instead of cold methanol, which was recommended for manual fixation.
2. For PSS, the fixative reagent is aliquoted into tubes or a 96-well plate and remains undisturbed before mixing with PBS, versus defrosting and immediately mixing with PBS manually. In both methods, the mixture is immediately applied to the tissue.
3. The final concentration of fixative reagent in PBS is 6.7% in the PSS method vs 2% in the manual process.

To demonstrate the automated method preserves robust fixation, a CD8 antibody was applied to a tonsil tissue sample and imaged three times on PCF (**Figure 3**). The first image was acquired after the first reporter PCF cycle (t=0; i.e., after background subtraction). Upon completion of cycle seven, the sample was stored at 4°C for 48 hours. The sample was subsequently imaged a second time on PCF (t=48h First Cycle) and again after completing cycle eight (t=48 Last Cycle).

Image analysis shows approximately equivalent gray value counts (standard deviations ~ 1% of the mean) for three cells imaged each time: Cell 1 = 50.0 ± 0.6; Cell 2 = 119 ± 2; Cell 3 = 85.7 ± 0.5. Thus, the automated PSS method maintained robust antibody fixation with no loss of antibody signal.

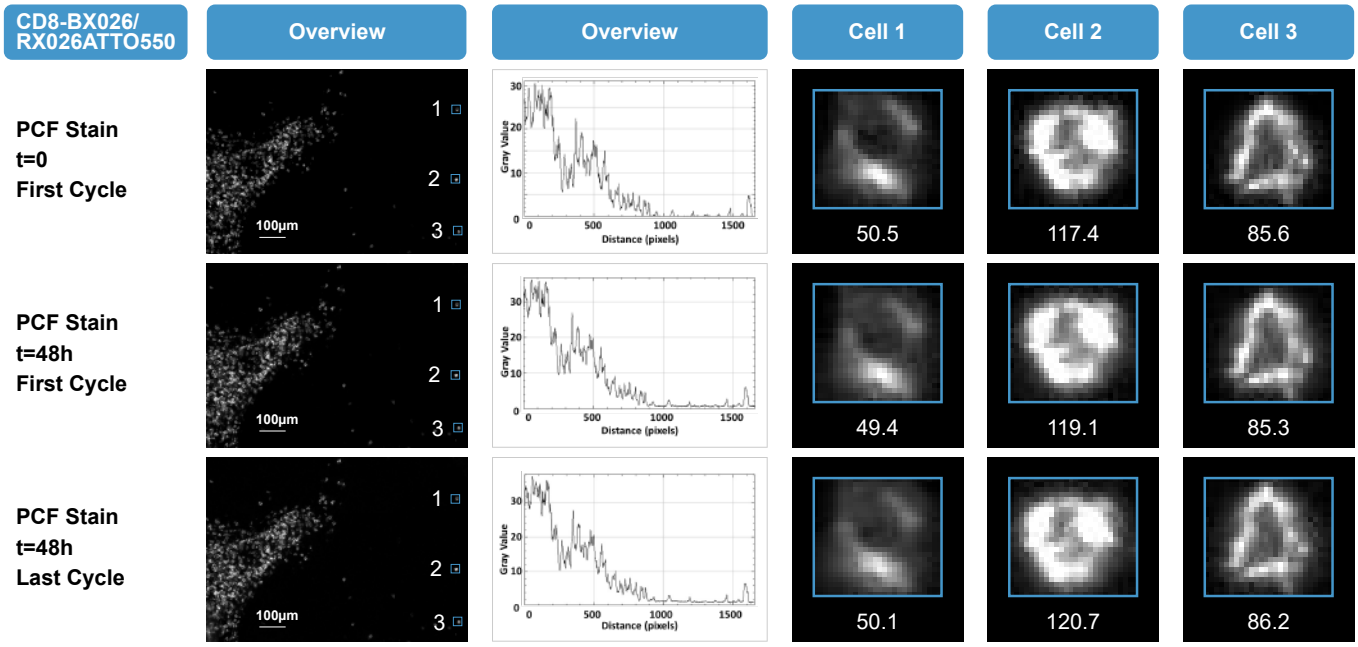


Figure 3. Fixation quality of PSS-Auto samples verifies method robustness. The antibody used was CD8-BX026/RX026ATTO550, Ratio 1:200, Exposure time: 250 ms.

Automated PCF specimen preparation achieves equivalent staining quality

Manual (**Figure 4A**) and PSS-Auto (**Figure 4B**) samples were sequentially imaged on the PCF using 15 total antibodies (three antibodies per cycle for five cycles, see **Supplementary Table 1**) that exhibit the spatial organization of the tonsil. Individual antibody stains are presented in **Figure S1**. Closer inspection of a central region flanking tonsillar crypts that features CD31-marked vasculature and CD3e-labeled T lymphocytes reveals similar staining and imaging quality for manually-processed samples (**Figure 4C**) as for the automated method on PSS (**Figure 4D**). While reporter quantitation was not carried out on these images, the PSS-Auto samples appear slightly brighter with good image contrast at the single-cell resolution.

Overall, the image quality of both preparations is comparable to the published tonsil by Akoya Biosciences employees and collaborators that utilized an ultra-high plex of over 100 antibodies.[3] Therefore, transferring the manual preparation method to automation on the PSS produces high-quality staining without significant demand on the researcher's time and attention.

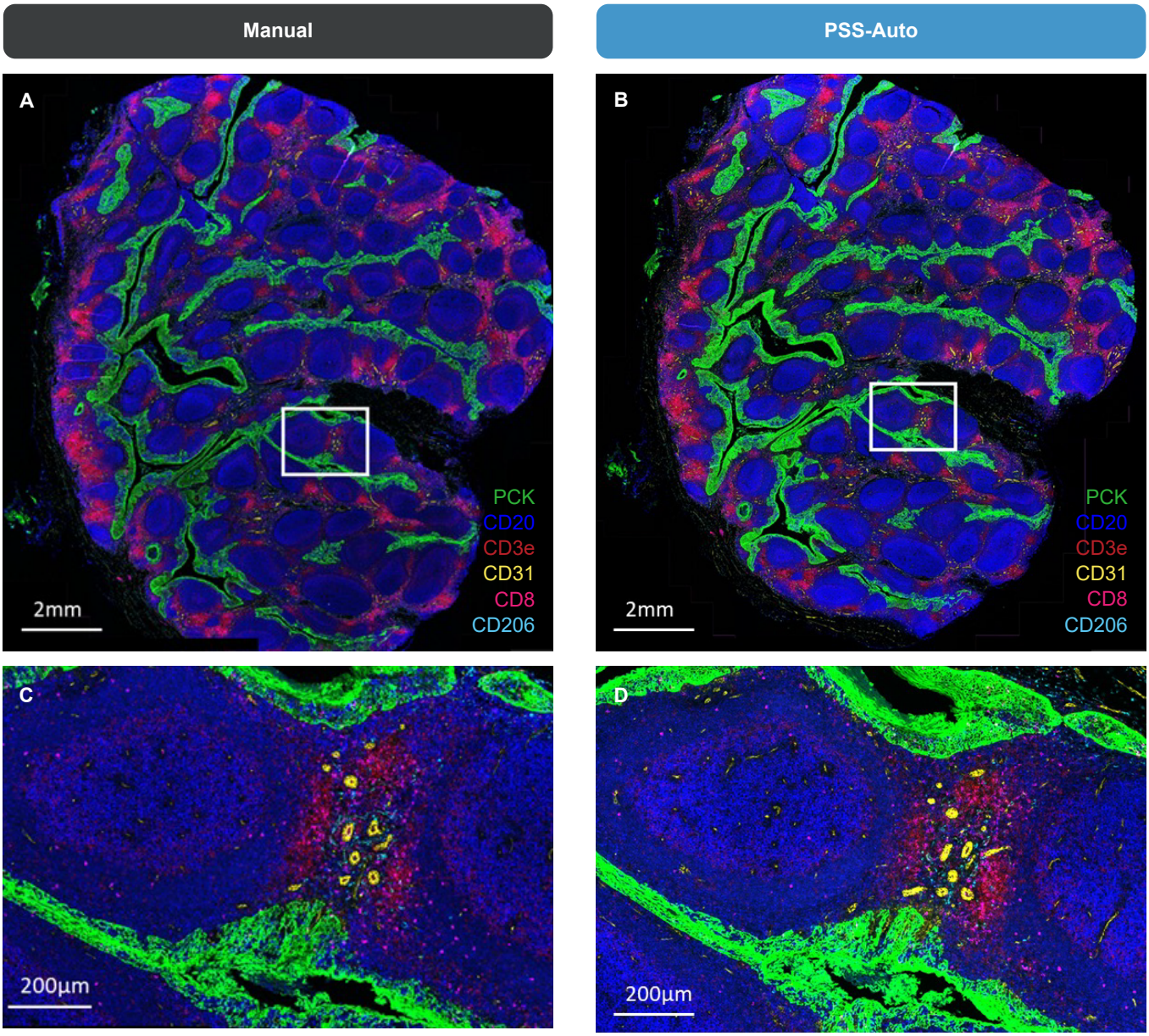


Figure 4. Automated staining on the PSS yields at least equivalent staining as a manual protocol. PCF imaging of FFPE human tonsil tissues stained with Pan-Cytokeratin (PCK, green), CD20 (blue), CD3e (red), CD31 (yellow), CD8 (magenta), and CD206 (cyan). The staining protocol was executed (A) manually or (B) automatically on the PSS and subsequently imaged on PCF. Similar regions for magnification are indicated with white boxes and displayed for samples stained (C) manually or (D) automatically on the PSS. PCK = Pan-Cytokeratin.

Automated staining procedure achieves uniform staining of two specimens on the same slide

Achieving uniform staining poses a challenge in manual sample preparation, given that the staining cocktail is applied to a corner of the tissue and parafilm is applied over the specimen to prevent dehydration. These hands-on steps invite inter-operator variability, exacerbated when two tissues are mounted on a single slide.

To demonstrate the uniformity of antibody application with the automated PSS procedure, slides containing two tonsil tissues were each stained manually or automated via PSS with a larger 27-plex panel (three antibodies per cycle for nine cycles, see **Supplementary Table 2**). Overall images for a subset of 12 antibody markers are presented in **Figure S2**, and magnifications of similar anatomical regions are shown in **Figure 5**.

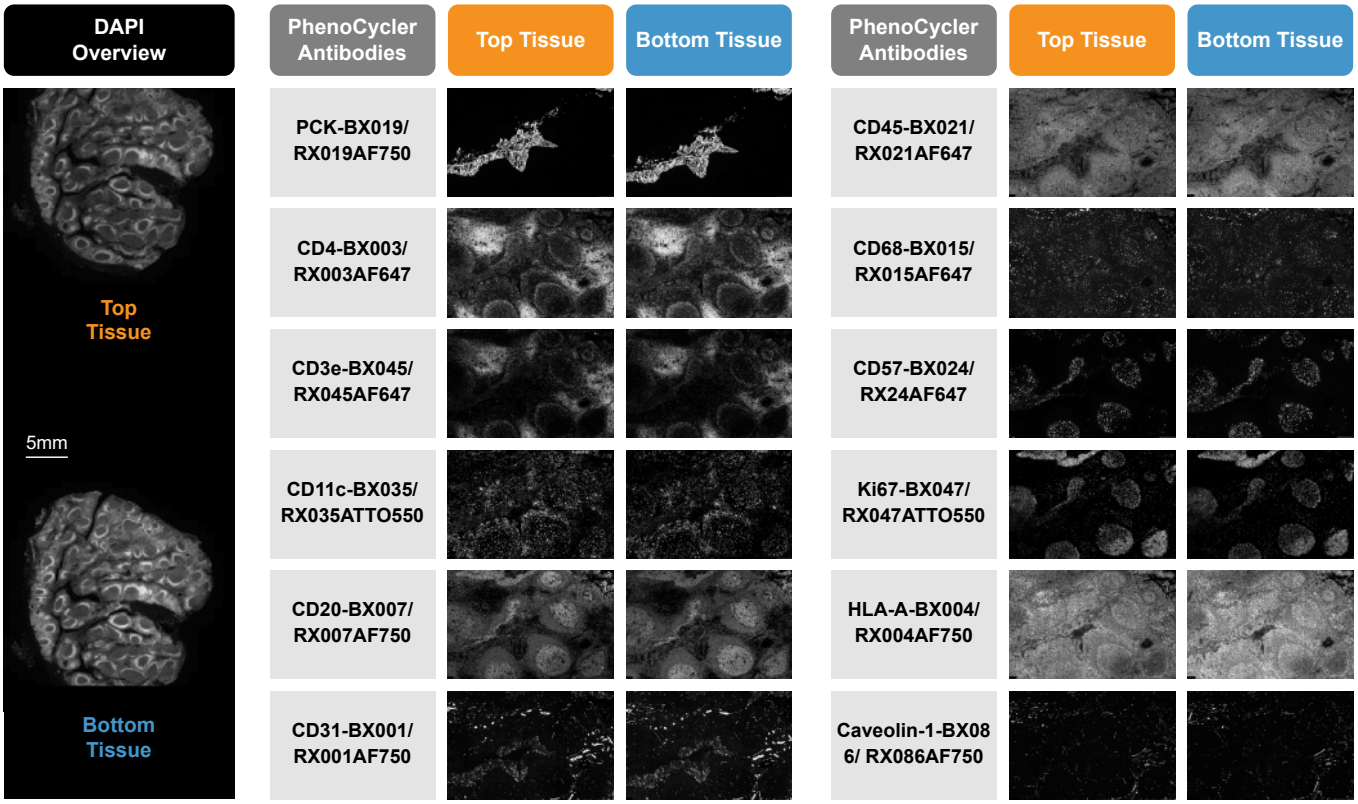


Figure 5. Automated PSS method yields uniform staining quality for both tissues of a doubly mounted sample. DAPI-stained overview (left) for commercially sourced FFPE tonsil tissue sections mounted on the same slide containing two specimens (labeled top and bottom). Images of each individual antibody staining corresponding to similar regions of the top and bottom tissue are indicated. Qualitatively, the staining is equivalent for both tissues.

The top and bottom tissues yielded visually equal staining quality for each antibody-reporter conjugate. No staining gradients or imaging artifacts were evident in the 12 antibody-reporter conjugates. Notably, no additional steps, reagents, or processing considerations were necessary for automating the staining of slides containing either a single or double-mounted specimen. Thus, the automated PSS method achieves uniform staining across a slide mounted with two tissues while further conserving reagents.

Automated staining procedure produces high-quality PCF imaging for a high-plex spatial immunophenotyping panel

To demonstrate comprehensive spatial phenotyping and characterization of the tissue microenvironment, the 27 high-plex panel was automatically applied to two human FFPE tonsil tissues mounted on the same slide. PCF imaging consisted of three antibodies per cycle for nine cycles.

Subsets of the 27-plex panel visualize the epithelium and vasculature of the tonsil (**Figure 6**). Equivalent staining quality across both tissues indicates uniform staining with the multiplexed assay (**Figure 6A**). Epithelial invaginations (**Figure 6B**) visualized by markers PCK and E-cadherin delineate expected tonsillar crypts that arise in response to antigens. In further magnifications of similar regions (**Figure 6C**), vasculature components Caveolin-1, CD31, and smooth muscle actin (αSMA) are clearly visible, collectively illustrating the demarcation of vasculature and epithelial components at single-cell resolution (**Figure 6D**).

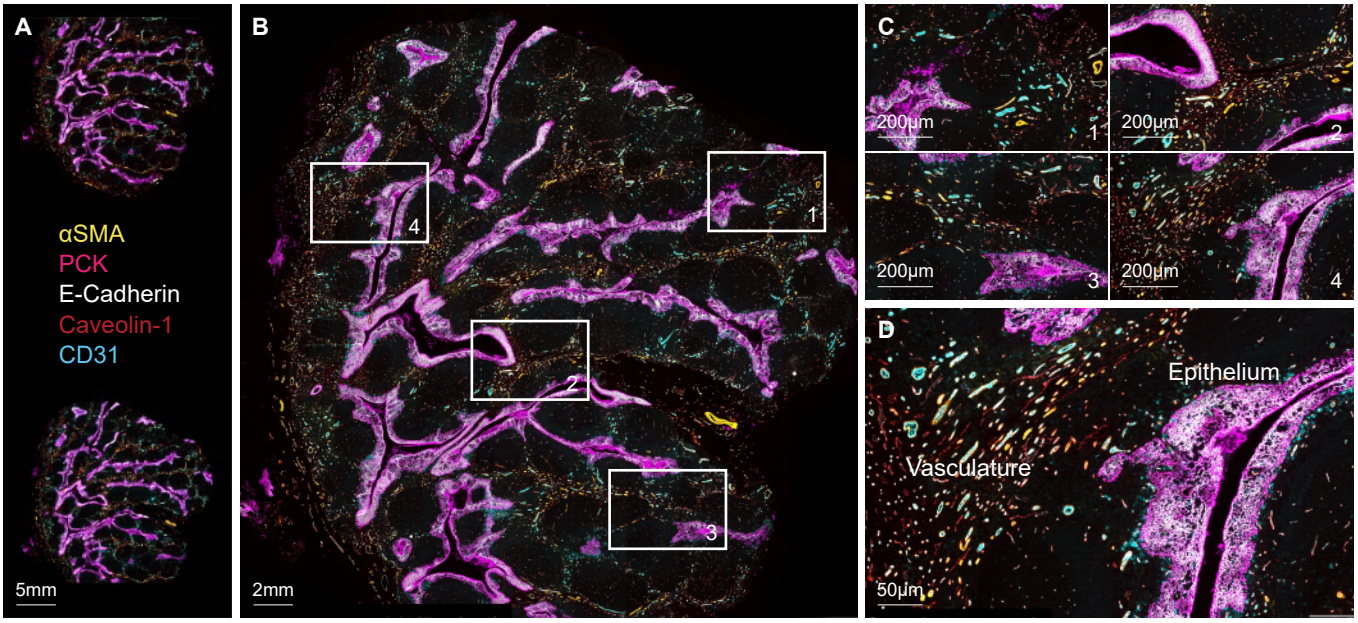


Figure 6. Whole-slide imaging of the automated staining protocol reveals epithelial and vasculature contexture. PCF imaging of FFPE human tonsil tissues autostained with αSMA (yellow), PCK (pink), E-cadherin (white), Caveolin-1 (red), and CD31 (cyan). (A) Overall slide with two tonsil specimens, (B) top tonsil with white boxes indicating (C) four magnified regions, and (D) Region 4 displaying clear differentiation of the vasculature and epithelium.

Multiplexed T-cell and NK cell contextures are presented in **Figure 7**. T-cell and NK cell-rich zones bordering tonsillar germinal centers are clearly distinguished in the four magnified regions (**Figure 7BC**). Localization and differentiation of CD4+ follicular helper T cells (Th), CD8+ cytotoxic T cells (Tc), and natural killer cells (NK) are evident with the auto-stained, multiplexed PCF imaging (**Figure 7D**). Furthermore, spatial regions containing higher levels of the immune checkpoint marker PD1, such as Region 1, can be distinguished from less abundant PD1 regions, such as Region 2.

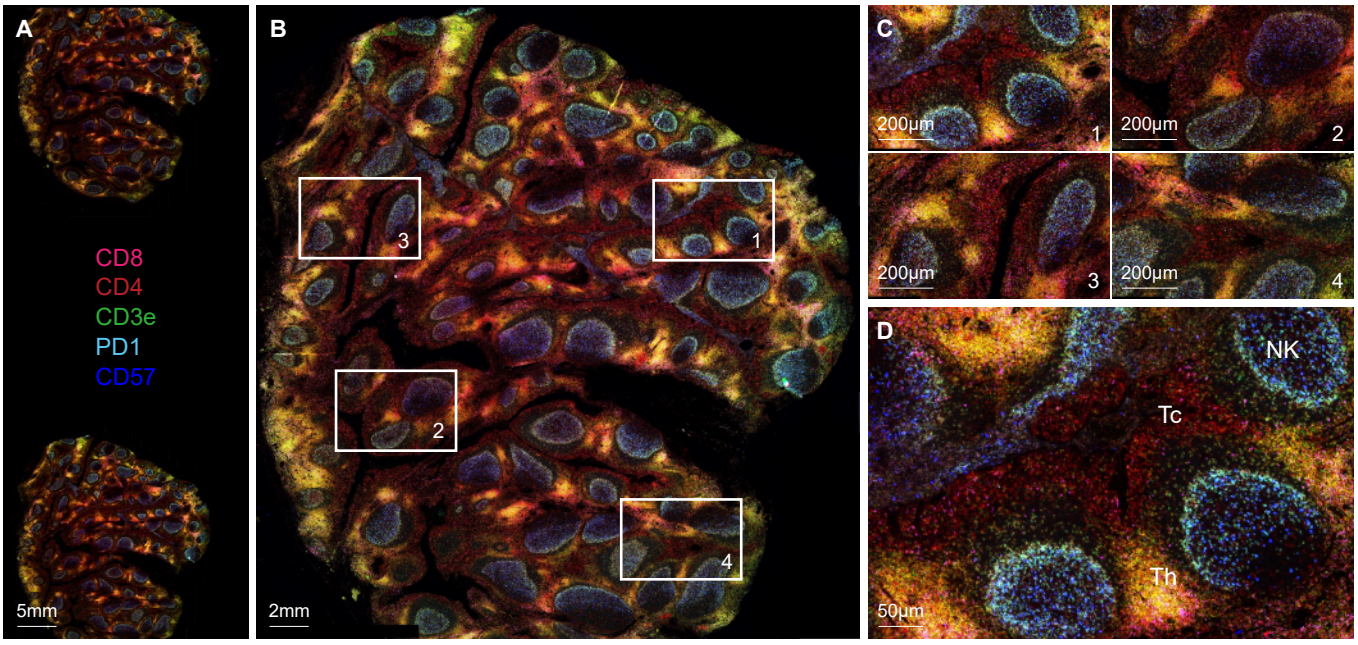


Figure 7. Whole-slide imaging of the automated staining protocol reveals lymphocyte contexture. PCF imaging of FFPE human tonsil tissues autostained with CD8 (magenta), CD4 (red), CD3e (green), PD1 (cyan), and CD57 (blue). (A) overall slide with two tonsil specimens, (B) top tonsil with white boxes indicating (C) four magnified regions labeled 1–4, and (D) Region 1 with spatial compartmentalization of cytotoxic CD8+ T cells (Tc), helper CD4+ T cells (Th) and natural killer cells (NK) indicated.

Multiplex imaging also elucidated myeloid cell spatial distribution (**Figure 8AB**). Dendritic cells (DCs) expressing CD11c were distinguished from macrophages, either M2-like macrophages expressing CD206 or CD68+/PDL1+ double-positive macrophages within human tonsil tissue (**Figure 8CD**). PDL1 expression is localized to epithelial regions and CD68+ macrophages. Taken together with the lymphocyte results, automated staining yields quality PCF imaging with potential utility in streamlining workflows in cancer immunotherapy and other applications that depend on spatial topography.

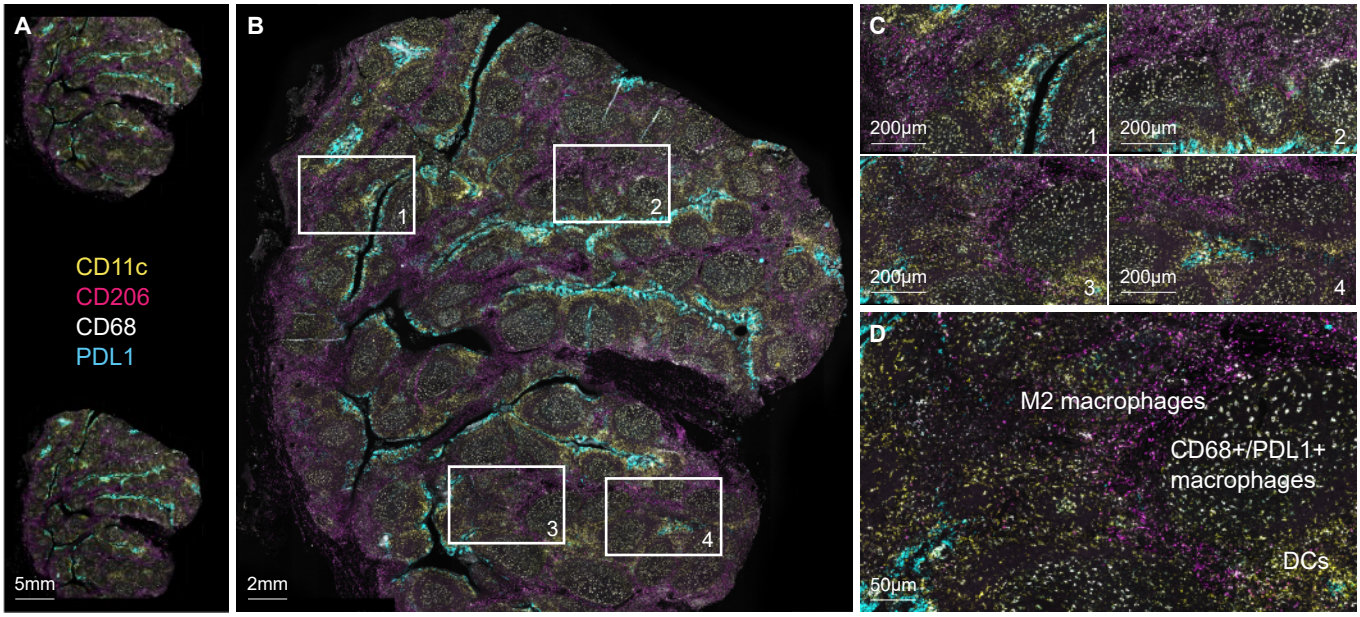


Figure 8. Whole-slide imaging of the automated staining protocol reveals myeloid cell contexture. PCF imaging of FFPE human tonsil tissues autostained with CD11c (yellow), CD206 (magenta), CD68 (white), and PDL1 (cyan). (A) overall slide with two tonsil specimens, (B) bottom tonsil with white boxes indicating (C) four magnified regopms labeled 1–4, and (D) Region 1 with spatial compartmentalization of M2 macrophages, double-positive CD68+/PDL1 macrophage, and dendritic cells (DCs) indicated.

A subset of markers from the 18-plex panel was chosen to visualize the tonsil structure and immune cell spatial distribution (**Figure 9**). The overall imaging (**Figure 9A**) and magnified region (**Figure 9BC**) clearly indicate epithelium (PCK and E-Cadherin) and vasculature (CD31), as well as the distribution of B-cells (CD20), T-cells (CD3e), NK cells (CD57), and macrophages (CD68) within the tissue.

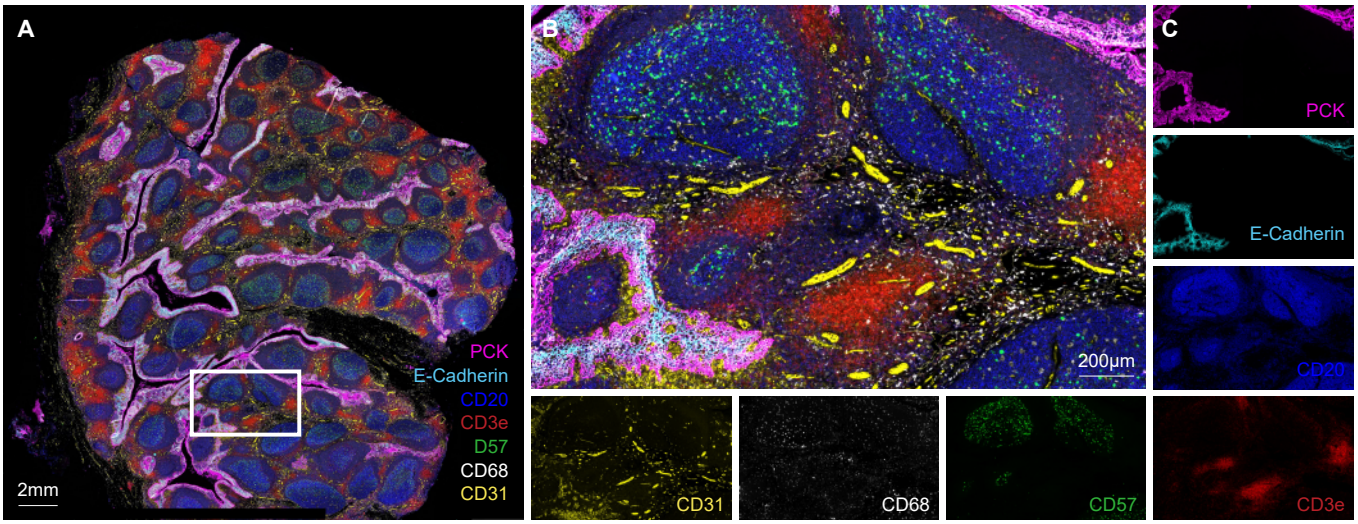


Figure 9. Whole-slide imaging of automated high-plex panel staining. (A) PCF imaging of FFPE human tonsil tissues (bottom tonsil shown) with a subset of markers visualized: PCK (magenta), E-cadherin (cyan), CD20 (blue), CD3e (red), CD57 (green), CD68 (white) and CD31 (yellow). The white box indicates the (B) magnified region displaying the seven markers, visualized individually in (C).

Automated H&E morphology staining after PCF imaging

The confirmation of structural topography with histological H&E staining is often accomplished after high-plex proteomic PCF imaging. PCF Flowcells are removed by incubating in xylene for 3–16 hours (here, 3 hours were sufficient). The capillary gap flowcell is then reassembled and placed in the PSS, and an H&E protocol is selected. Automated H&E staining yielded high-quality morphological imaging that corresponds well with the high-plex PCF imaging, revealing the expected tonsillar crypts, lymph nodules, connective tissue, and the germinal center (**Figure 10**). Thus, routine histology staining accompanying PCF imaging can also be automated on the PSS.

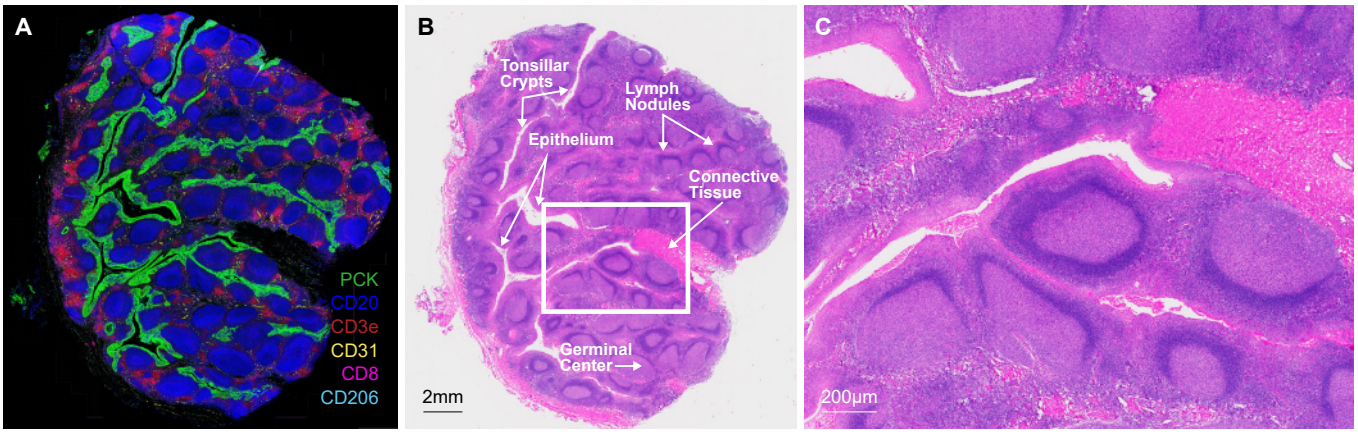


Figure 10. Hematoxylin and eosin (H&E) morphology staining automation on the PSS after PCF imaging. FFPE human tonsil tissue was imaged on (A) PCF with a subset of markers visualized: PCK (green), CD20 (blue), CD3e (red), CD31 (yellow), CD8 (magenta), and CD206 (cyan). (B) Hematoxylin (pink) and eosin (purple) autostaining on the PSS after PCF and removal of the Flowcell. Major tonsillar features are indicated; the white box denotes the (C) magnified region.

Operator time savings with PSS automation

We have described the PCF antibody and H&E staining workflow automation; however, the PSS can also automate deparaffinization, epitope retrieval, and reporter plate preparation. As described in the PCF STAR methods publication, the entire sample preparation workflow requires ~20 hours, which includes an overnight 4˚C antibody incubation.[4] Much of the remaining ~8 hours requires precise hands-on timing on behalf of the researcher and, thus, additional planning to account for the individual steps and timing of the overnight incubation.

With automation, hands-on time is minimized to 3 primary interventions totaling 30–40 minutes: application of CoverPads to the specimen slides, assembly of the S12 module with slides, and choosing assay parameters using the touchscreen (**Table 2**). The researcher does not need to be present for the rest of the assay time (i.e., walkaway time) and can plan accordingly for additional overnight runs or room-temperature incubations that reduce the overall assay time. Furthermore, eliminating the need for manual pipetting and parafilm application substantially reduces the requirement for human intervention and thus reduces assay variability.

Table 2. Time savings and reductions in error potential associated with automating the PCF staining protocol.

Manual Protocol				Spatial Station Automation			
Step	**Average Manual time**	**Level of human intervention**	**Observation and outcome**	**Step**	**Average Manual time**	**Level of human intervention**	**Observation and outcome**
1	2-3 hours	High	Inconsistent between researchers/labs.	1	20-30 mins	Low to none	Superior results, repeatable, and reproduceable.
2	3-4 hours	Very High	Varies between researchers.	2	20-30 mins	Very low	Superior outcome, repeatable, and reproduceable.
3	1 hour	High	Varies between researchers.	3	20-30 mins	Low to none	Consistent, high accuracy, and reproducibility.
4	1 hour	High	Varies between researchers.	4	10 mins	Very low	Consistent, high accuracy, and reproducibility.

Conclusions

In this report, we automated the specimen staining protocol for PCF imaging and validated the resulting method. Antibody staining on the PSS is robust and reliable, as evident by the absence of signal loss that would result from inconsistent fixation. The technique yields uniform slide staining with two specimens and equivalent, if not better, staining quality than the manual protocol. The automated application of a high-plex panel exploring tissue spatial structure and immuno-phenotyping demonstrates the method's utility for spatial biology applications. Removal of the PCF Flowcell and subsequent automated H&E staining further validate morphology quality control as part of the workflow. End-to-end automation of sample processing using the baking, dewaxing, and epitope retrieval functionality of the PSS (and Omni-Stainer) can also be accomplished, providing a standardized PCF pre-imaging workflow that reduces operator hands-on time and variability.

Supplementary Figures

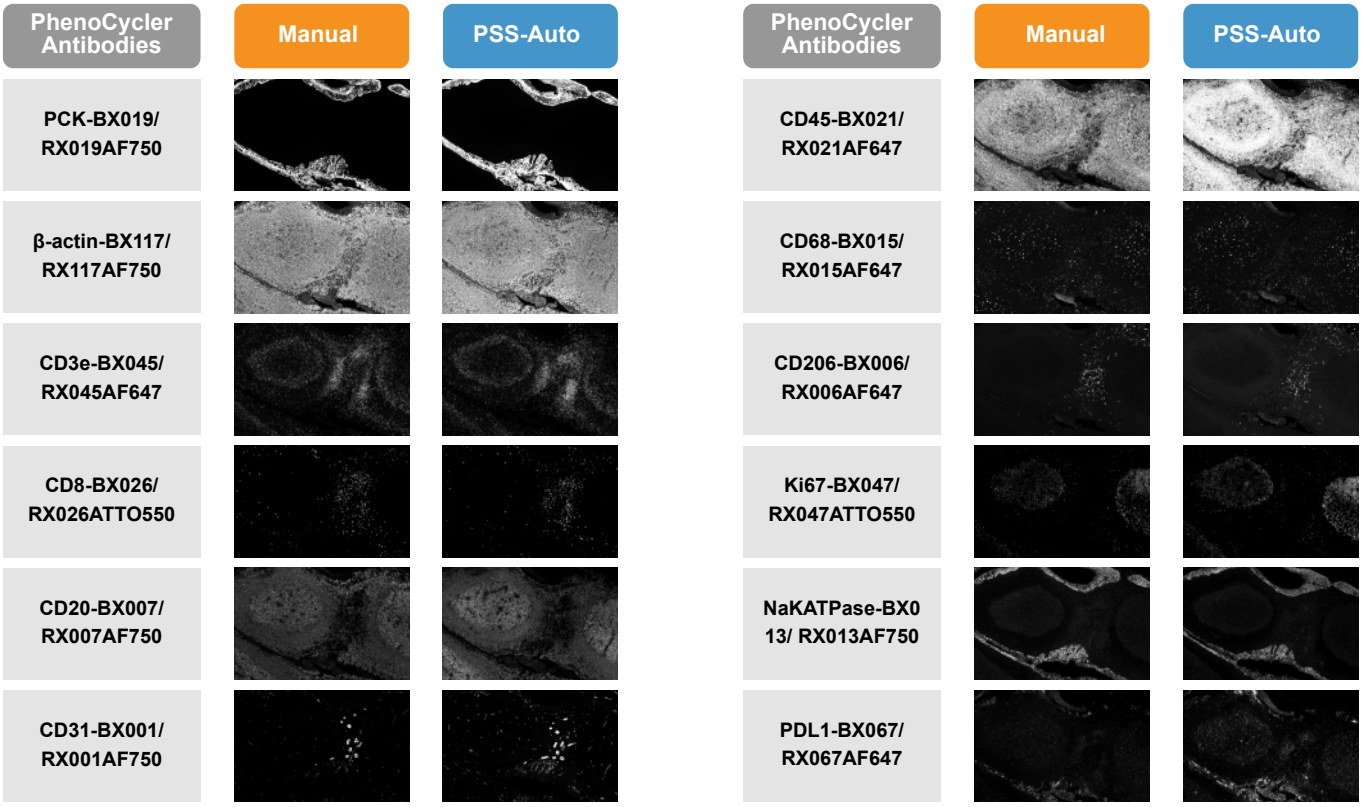


Figure S1. Individual antibody images of tonsil regions comparing Manual and PSS-Auto staining protocols. The Phenocycler antibody-reporter conjugates for each stain are indicated for samples processed manually (salmon) or automated on the PSS (blue). See Figure 4 for the magnified region location.

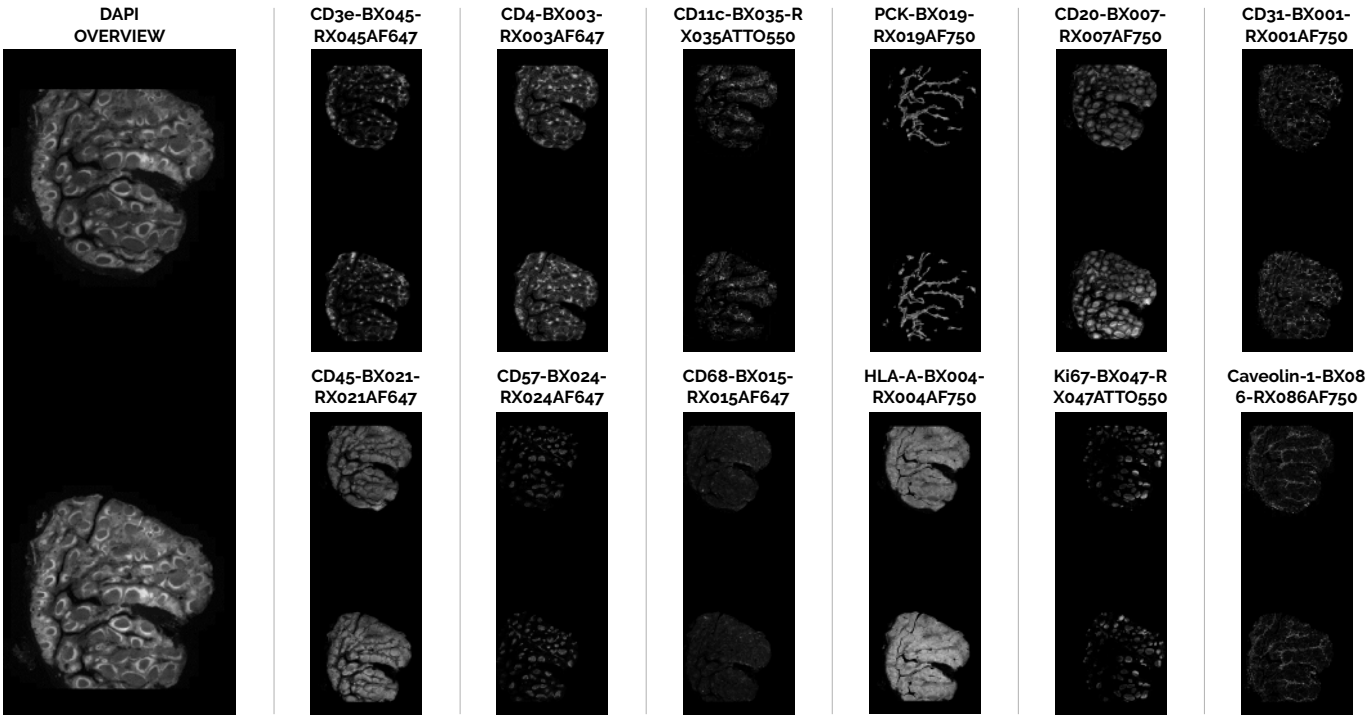


Figure S2. Overall PSS-Auto antibody images of two tonsil specimens on a single slide. The Phenocycler antibody-reporter conjugates for each stain are indicated for samples processed on the PSS. See Figure 5 for magnified region images.

Supplementary Table 1. List of 15-panel antibodies, barcodes, and reporters used in the PSS automation validation.

Cycle No.	Antibody	Barcode	Reporter
1	Blank Blank Blank		
2	CD8a CD68 CD20	BX026 BX015 BX007	ATTO550 AF647 AF750
3	CD45RO CD206 CD31	BX017 BX006 BX001	ATTO550 AF647 AF750
4	CD14 PDL1 NaKATP	BX037 BX067 BX013	ATTO550 AF647 AF750
5	CD11b CD3e b-actin	BX002 BX045 BX117	ATTO550 AF647 AF750
6	Ki67 CD45 PCK	BX047 BX021 BX019	ATTO550 AF647 AF750
7	Blank Blank Blank		

Supplementary Table 2. List of 27-panel antibodies, barcodes, and reporters used in the PSS automation validation.

Cycle No.	Antibody	Barcode	Reporter
1	Blank Blank Blank		
2	CD8a CD68 CD20	BX026 BX015 BX007	ATTO550 AF647 AF750
3	CD45RO CD206 CD31	BX017 BX006 BX001	ATTO550 AF647 AF750
4	CD14 PDL1 αSMA	BX037 BX067 BX013	ATTO550 AF647 AF750
5	CD11c CD3e b-actin	BX035 BX045 BX117	ATTO550 AF647 AF750
6	ICOS PD1 Vimentin	BX054 BX046 BX022	ATTO550 AF647 AF750
7	E-cadherin Foxp3 Caveolin-1	BX014 BX031 BX086	ATTO550 AF647 AF750
8	CD15 CD4 HLA-A	BX023 BX003 BX004	ATTO550 AF647 AF750
9	GranzB CD57 CD79A	BX041 BX024 BX090	ATTO550 AF647 AF750
10	Ki67 CD45 PCK	BX047 BX021 BX019	ATTO550 RX021AF647 AF750
11	Blank Blank Blank		

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