High-quality, automated, and scalable solutions for FFPE sample dewaxing and antigen retrieval protocols

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Abstract

Spatial biology is a rapidly advancing field with enormous potential to uncover biological processes and enhance biomarker discovery in therapy development. Both legacy and cutting-edge visual and molecular techniques are often performed on formalin-fixed, paraffin-embedded (FFPE) specimens, which often involve manually performed processing steps to remove wax and expose epitopes to staining reagents. Inherent inconsistencies with these laborious workflows demand researcher attention and can degrade the final data quality. Here, we discuss the common protocols associated with FFPE specimen processing and propose an alternative, standardized, and automated approach that improves throughput, consistency, and operator productivity.

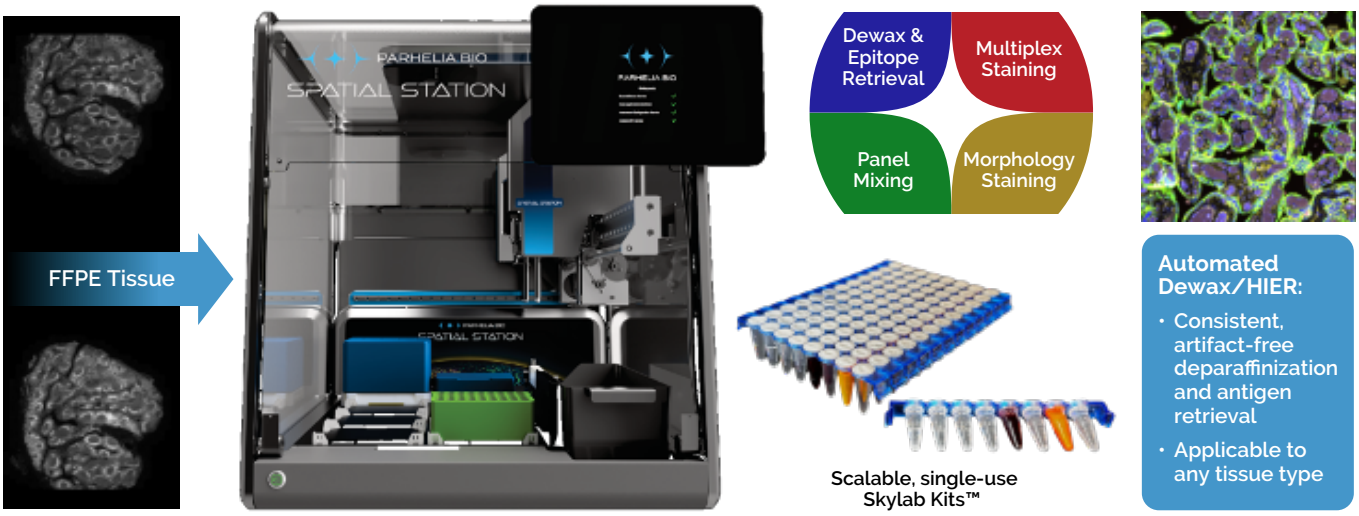


Figure 1. Graphical abstract overview of FFPE sample processing on the Parhelia Spatial Station™.

Preserving tissue specimens for downstream analysis

Spatial biology describes how biological systems interact in space and time with molecular and cellular detail.[1] To protect tumor resections, biopsies, and other valuable samples from degradation, the specimens often undergo formalin fixation, dehydration, and paraffin embedding protocols, resulting in FFPE specimens.[2] While these processes preserve and support tissues for *ex vivo* analysis, they also introduce artifacts that can complicate the analysis, depending on the technique, and lead to deviations from *in situ* biology.

Removing paraffin: dewaxing and rehydration

Paraffin is a hydrophobic wax that coats tissue specimens and prevents dehydration. Before any staining procedure, including hematoxylin and eosin (H&E), staining with antibodies (e.g., multiplex immunofluorescence or CODEX technology), and in situ hybridization (ISH), the paraffin must be removed entirely (i.e., dewaxed) for the staining chemicals or molecular probes to penetrate the tissues. High-quality dewaxing completely removes the paraffin while preserving tissue morphology and promoting antibody binding. Conversely, incomplete dewaxing complicates staining interactions and can introduce artifacts, such as autofluorescence, that negatively impact data analysis.

The typical dewaxing procedure involves melting the paraffin by baking the specimen at 60˚C for 30 minutes to overnight, which improves tissue attachment to the glass. Next, incubation in xylene or its substituents (naphthenic solvents, d-limonenes, and aliphatic hydrocarbons) dissolves the wax. Subsequently, the xylene is removed with a xylene: ethanol gradient, followed by an ethanol: water gradient so that the specimen is left in pure water.

While xylene is by far the most common choice of dewaxing agent, it is considered toxic, teratogenic, and carcinogenic, necessitating associated steps to be performed in a chemical hood. Less toxic choices are available; however, researchers insist they are comparably inefficient at dewaxing. The number of washes and concentrations of reagents are antigen and tissue-dependent, requiring knowledge on behalf of the scientist and substantial manual processing. Finally, the standard 15-minute xylene dewaxing protocol was shown using Raman spectroscopy to only partially remove paraffin.[3] Therefore, the spatial biology community continues to demand a safe and high-performing alternative dewaxing reagent.

Removing formaldehyde: Heat-induced epitope retrieval (HIER)

Formalin (the liquid form of formaldehyde) preserves tissue morphology and cellular details by forming methylene bridges ("crosslinking") between primary amino groups found ubiquitously on proteins and other biomolecules. Consequently, these chemical modifications can reduce the accessibility of target epitopes, masking them from staining antibodies or probes. Formaldehyde-induced crosslinking is especially problematic for RNA molecules, resulting in strand fragmentation.[4]

Most commonly, the unmasking of stain binding sites (i.e., antigen retrieval) is accomplished by heating the specimens in a buffered solution using heat-induced epitope retrieval (HIER) protocols specific to the tissue type and antibody/probe being used.[5] Common buffers range from mildly acidic (10 mM sodium citrate, pH 6), mildly basic (1 mM EDTA, pH 8), to alkaline (1 mM Tris/EDTA, pH 9) conditions.[2] Achieving temperatures of 95–100˚C is carried out using a plethora of laboratory and kitchen appliances, including hotplates, microwave ovens, pressure cookers, and vegetable steamers.[5] Chelation of bound calcium ions is believed to contribute to the mechanism of HIER, given the success of citrate and EDTA-containing buffers.[5]

The choice of buffer and heating vessel depends on the tissue under investigation and the researcher's experience, complicating the standardization of antigen retrieval methods both within and across laboratories. There is no agreed-upon, "universal" HIER buffer, although EDTA is most effective on "over-fixed" specimens.[6] Conversely, EDTA can distort cellular and nuclei morphology, while alkaline pH may cause section loss.[7] In choosing a heating apparatus, the researcher generally faces with a choice between incubation time and even heating distribution; however no conventional mechanism provides both even distribution and fast incubation.[5]

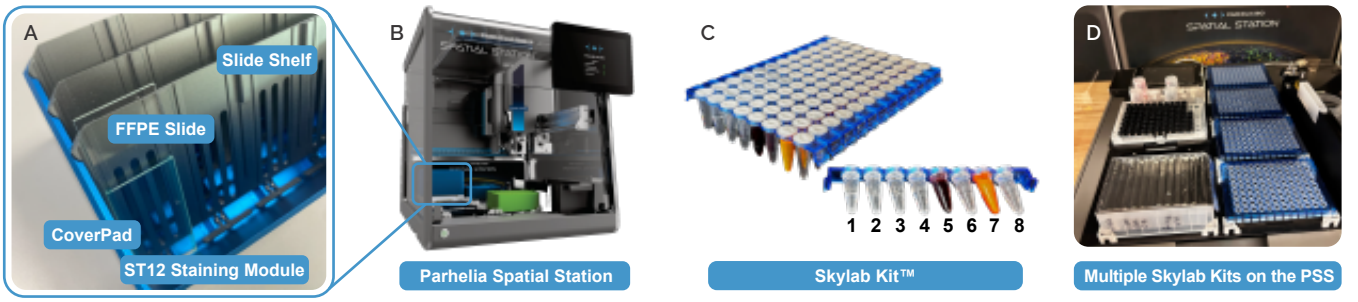
Protease-induced epitope retrieval (PIER) represents an alternative to HIER, relying on the activity of peptidases (e.g., pronase, pepsin, ficin, trypsin, and proteinase K) to unmask antibody-binding epitopes rather than extreme heating and chelation.[2] PIER was the method of choice prior to the development of HIER techniques; however, enzymatic digestion can degrade the epitope, making it unrecognizable by certain antibodies. Achieving sufficient proteolysis requires optimizing the reaction conditions, including enzyme concentration, temperature, and time, as well as validation following antibody staining.[2]

Table 1. Summary of challenges with the dewaxing, rehydration, and antigen retrieval

Challenge	Dewax & Rehydration	Antigen Retrieval
Consistency of methodology	15-min xylene incubation may be inefficient Number of washes may vary between operators and specimens	Choice of heating apparatus and buffer varies from lab-to-lab and among different specimen types
Operator safety	Xylene-containing steps must be performed in a chemical fume hood	Some heating systems risk burn or explosion injuries
Operator training and hands-on time	Requires attention and constant pipetting during washes Chemical fume hood requires different skill sets and training Steps performed in and out of the fume hood	Requires attention and constant pipetting during washes Choice of buffer pH is antigen-dependent and may require optimization
Consistency in data quality	Incomplete dewaxing significantly and negatively impacts downstream data quality	Antigen retrieval optimization may differ from lab-to-lab and may depend on subtle procedural differences

Automated dewaxing and de-fixing of FFPE specimens

The Omni-Stainer™ and Spatial Station™ are instruments developed by Parhelia Biosciences to automate the specimen processing and staining steps upstream of spatial analysis. By precisely controlling temperature, applying reagents via the capillary gap method, and using a Parhelia-developed non-toxic dewaxing reagent, operators can achieve hands-free and efficient dewaxing, antigen retrieval, and enhanced staining quality compared to other contemporary methods. Notably, the dewaxing and epitope-retrieval reaction conditions can be optimized and standardized on either automated system, regardless of the choice of dewaxing agent, HIER, or proteolysis.



Kit	1	2	3	4	5	6	7	8
Dewax/HIER	Parhelia Dewax Reagent	100% EtOH	70% EtOH	TBST	HIER buffer	HIER buffer	TBST	TBST
Dewax/H&E				Rnase/DNase free water	Hematoxylin	Bluing reagent	Eosin	100% EtOH

Figure 2: Automated slide processing, staining, and scaling on the Parhelia Spatial Station (PSS). (A) Assembly of the ST12 Staining Module, (B) Parhelia Spatial Station, (C) Skylab Kit, each of which contains 12 reactions (8 vials each). Dewax/H&E Kit shown as an example; note the Dewax/HIER Kit contains all clear liquids, (D) Slide processing throughput can be increased by simply scaling the number of kits.

Procedure

Experiments

All immunofluorescence studies were performed at the National Cancer Institute (NCI) in the Spatial Imaging Technology Resource (SpITR) laboratory. The immunohistochemistry (IHC) study comparing Skylab Dewax/HIER with manual methods was performed in the Baxter Laboratory in Stem Cell Biology (Prof. Garry Nolan's lab) at the Stanford University School of Medicine.

Preparing the Instrument

1. Prepare a flow cell for each slide by removing the coverslips and securing a CoverPad directly over the specimen (dry loading).

2. Load dry flow cells into the S12 module and place the S12 module on top of the Thermal Module, covering it with the insulating sheath.

3. Choose the appropriate automated protocols for sample processing:

    - Dewaxing
    - Rehydration
    - HIER
    - (optional) Antibody, probe, or chromophore staining

*Note: the Parhelia Omni-Stainer can also accomplish these experimental steps but was not evaluated in this study.

Automated Specimen Processing

The Omni-Stainer or Spatial Station automatically accomplishes the following steps according to the protocol selected. The volumes of reagent applied (e.g., dewaxing reagent, alcohol washes, and staining reagents) are set to 150 µl but can be easily adjusted according to the protocol.

For precise and reproducible results, choose Parhelia Skylab™ Kits. These kits include pre-aliquoted reagents in eight-tube strips, where one strip comprises all the reagents necessary for one sample. Skylab Kits are available for Dewax+HIER and Dewax+H&E (hematoxylin plus eosin staining).[8]

4. Baking: Evenly heats the samples to 65˚C or any desired temperature or incubation time.^

5. Dewaxing: At 72˚C, three volumes of 100 µl or 2x150 µl of dewaxing reagent are delivered to each flow cell via laminar flow generated by capillary action.

6. Rehydration: At 50˚C, wash the samples twice each with 150 µl of 100% EtOH, 95% EtOH, 70% EtOH, and PBS to achieve a uniform rehydration.

7. HIER: Selected antigen retrieval buffer is added at 50˚C, followed by the thermal block ramping up to the indicated temperature and time (typically 98˚C for 40 minutes). The system achieves consistent and uniform heating in an enclosed space while topping off the flow cell with additional buffer to prevent the sample from drying out.

8. Cooling: Samples are cooled to room temperature for additional washes, subsequent staining assays, or storage temperature (4–8˚C).

9. Antibody staining: Various antibody or chromogen staining protocols can be customized and executed automatically.

Imaging

For this study, specimens were imaged sequentially on several different platforms, as indicated: GeoMx DSP (Nanostring Technologies, Bruker Spatial Biology), PhenoCycler Fusion (Akoya Biosciences, Inc.), and Axioscan 7 (Zeiss) using the same exposure and signal intensity settings for each workflow.

Specimens

FFPE tissue from human colorectal cancer and colon adenocarcinoma was used to compare the PSS automated method, the classical manual method, and another automated method featuring the Leica Bond instrument. The various antigen retrieval buffers in both bulk and Skylab Kit formats were compared using FFPE tissue from human mesothelioma tissue. FFPE human tonsil tissue was used to compare manual and PSS-Skylab IHC staining protocols. Successive tissue sections were utilized to minimize spatial variability between the tested methods. FFPE preserved 23 Core Normal Human Tissue Microarrays (Bio SB, Santa

^Note: many labs prefer baking slides at 60˚C in a dedicated incubator. Baking in the PSS should be performed at 65˚C. In this study, baking was performed external of the automation platforms at 60˚C for 1 hr.

Barbara, CA) were employed to compare the performance of the automated processes in various tissue types. FFPE human colorectal cancer liver metastasis and lymph node tissues were used to demonstrate Skylab H&E staining quality. **Table 2** summarizes the baking, dewaxing, rehydration, and epitope retrieval among manual and automated techniques.

Table 2. Summary comparison of the methods used in this study.

Step	Manual	Leica Bond ER2	PSS pH9	PSS ER2	PSS G-buffer
Baking	60˚C for 60 min in a dedicated oven				
Dewax	Xylene 72˚C	Leica dewax 72˚C	Parhelia dewax 72˚C		
Rehydration	100/90/70/50/ 30% EtOH, 5 min each	100/70% EtOH, 5 min each	100/70% EtOH , 5 min each		
HIER	DAKO pH9, 20 min at 100˚C in pressure cooker	ER2 buffer, 20 min at 100˚C	DAKO pH9, 40 min at 98˚C	Leica ER2 buffer, 40 min at 98˚C	Proprietary Parhelia buffer, 40 min at 98˚C

Staining

Unless otherwise noted, all staining was performed automatically on the PSS.[9] Exceptions where manual staining was conducted included the comparisons using colon adenocarcinoma tissue, the human tissue microarray, and the immunohistochemistry staining. See **Table S1** for a list of antibodies and fluorophores used in this study.

Results

Automated processing of colorectal cancer tissue on PSS compares well with manual or automated methods on Leica Bond

To demonstrate dewax and antigen retrieval quality on the PSS, we used tissue sections of colorectal cancer. Pairs of tissue sets were processed and subsequently imaged on different days, with examples from Day 2 are shown in **Figure 3**. For epithelial markers Pan-Cytokeratin (PCK) and E-cadherin, the staining quality was consistently quite comparable among the four methods. However, for the leukocyte marker CD45 the manually processed specimens were noticeably weaker than the automated methods on both Day 1 and Day 2 **(Figure 3B)**. The lower staining quality may be due to the xylene treatment affecting antibody binding. When the experiment was repeated and CD45 was replaced with CD8a, we found that the manually processed images produced stronger epithelial staining with sufficient contrast for both automated methods. CD8a cytotoxic T cells were easily and comparably resolved regardless of the preparation method **(Figure 3C)**.

These results with colorectal cancer tissue indicate that PSS automation of dewaxing and epitope retrieval produces staining quality comparable to an established automated method and traditional manual processing. The Parhelia dewax solution, and the proprietary Leica dewax solution seem to enhance immune cell staining quality compared to classical xylenes. The automated methods offer the advantage of significantly reduced hands-on time, while the PSS system having almost no dead volume, consumes fewer buffers and antibodies than the Leica system.[9]

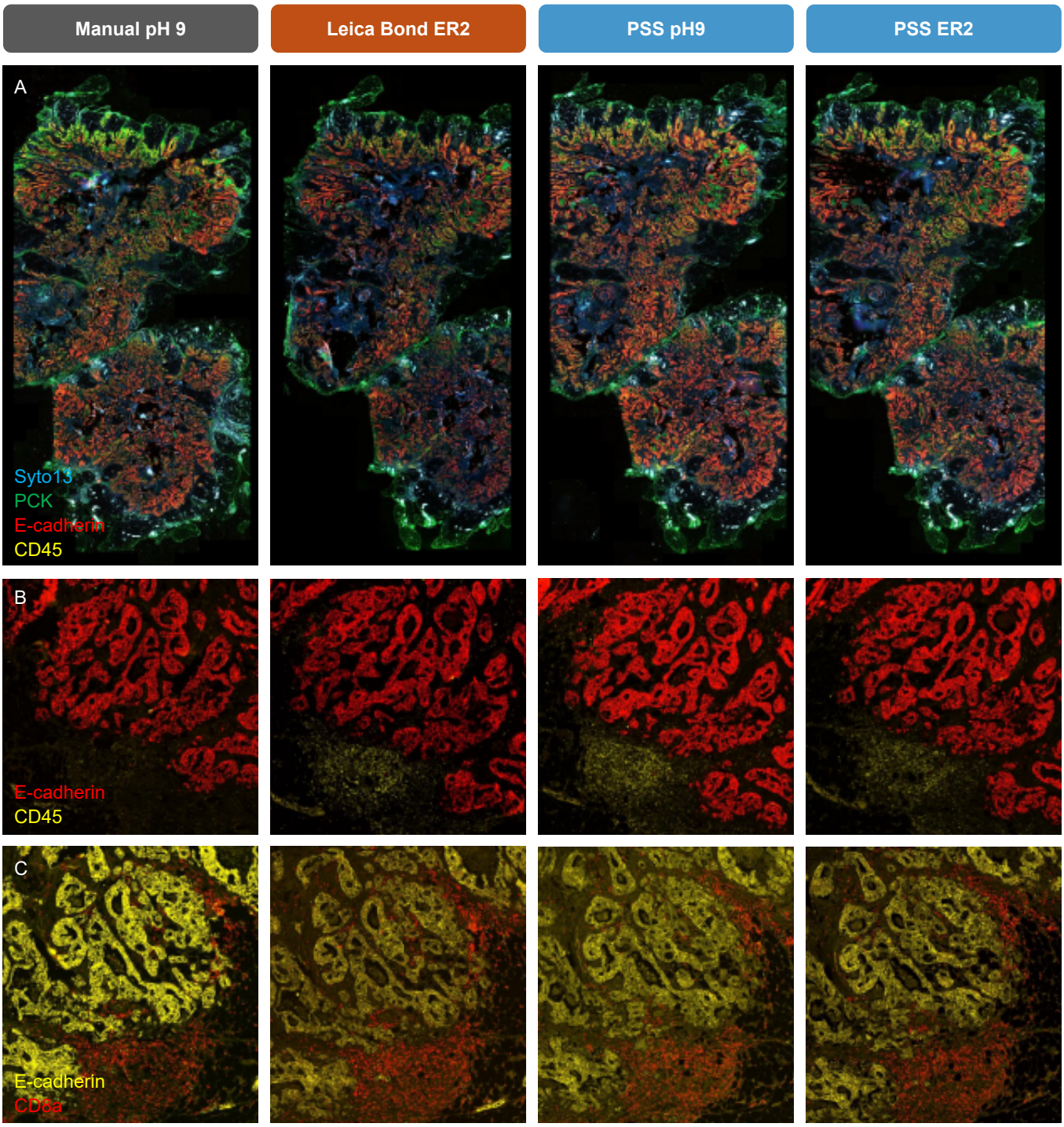


Figure 3: Comparison of colorectal cancer FFPE processing between manual and automated methods. (A) Composite images of stained tissues showing Syto13-stained nuclei (blue) and antibody stains PCK (green), E-cadherin (red), and CD45 (yellow). Zoomed-in areas displaying E-cadherin and (B) CD45-stained cells (yellow) and (C) CD8a-stained cells (red). Imaging was performed on a GeoMx DSP instrument.

To further compare the four dewax-HIER methods, a different FFPE-preserved colon adenocarcinoma tissue was processed and imaged on another day. PCK and CD8a were directly labeled manually using standard multiplex immunofluorescence methodology. CD8a staining is sensitive to high-temperature HIER conditions and thus serves as a good marker for dewax-plus-antigen retrieval evaluation. Alkaline buffers

from Dako (pH9) and Leica (ER2) were tested to demonstrate buffer compatibility. Overall, the PSS methods consistently achieved high-quality staining quality that clearly differentiates the epithelial tissue (PCK-labeled) from cytotoxic CD8+ T cells in a manner roughly equivalent to manual techniques and those of Leica Bond automation **(Figure 4)**.

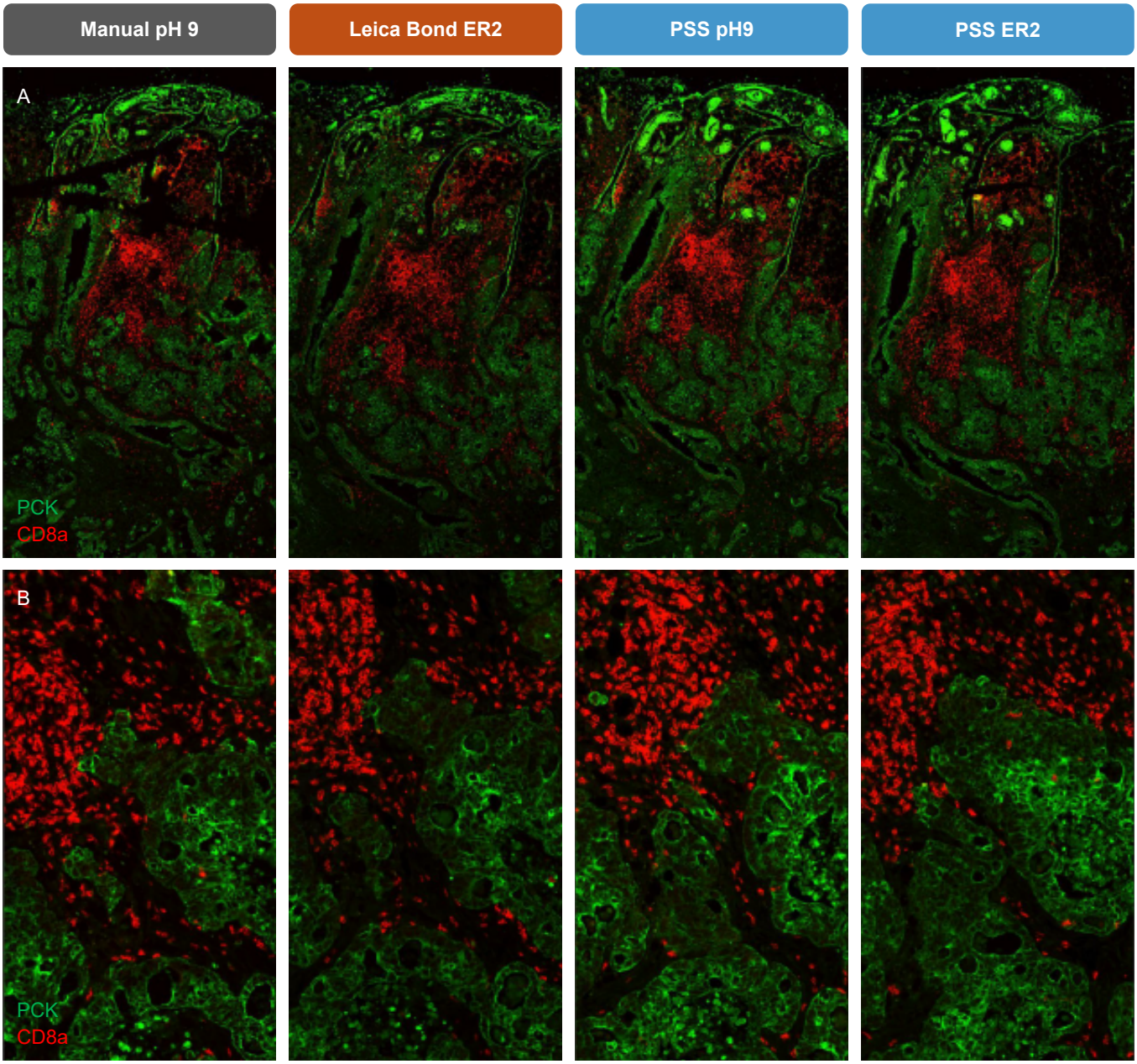


Figure 4: Comparison of colorectal adenocarcinoma FFPE processing between manual and automated methods. (A) Overview and (B) zoomed-in. Antibody stains include PCK (green) and CD8a (red). Imaging was performed on a GeoMx DSP instrument.

Automated dewaxing and antigen retrieval processing of tissue microarrays

To compare the performance of automation platforms between the PSS and the Leica Bond, we evaluated several healthy human tissues using a microarray format. Staining was performed manually with the same antibodies and buffers for a more direct comparison.

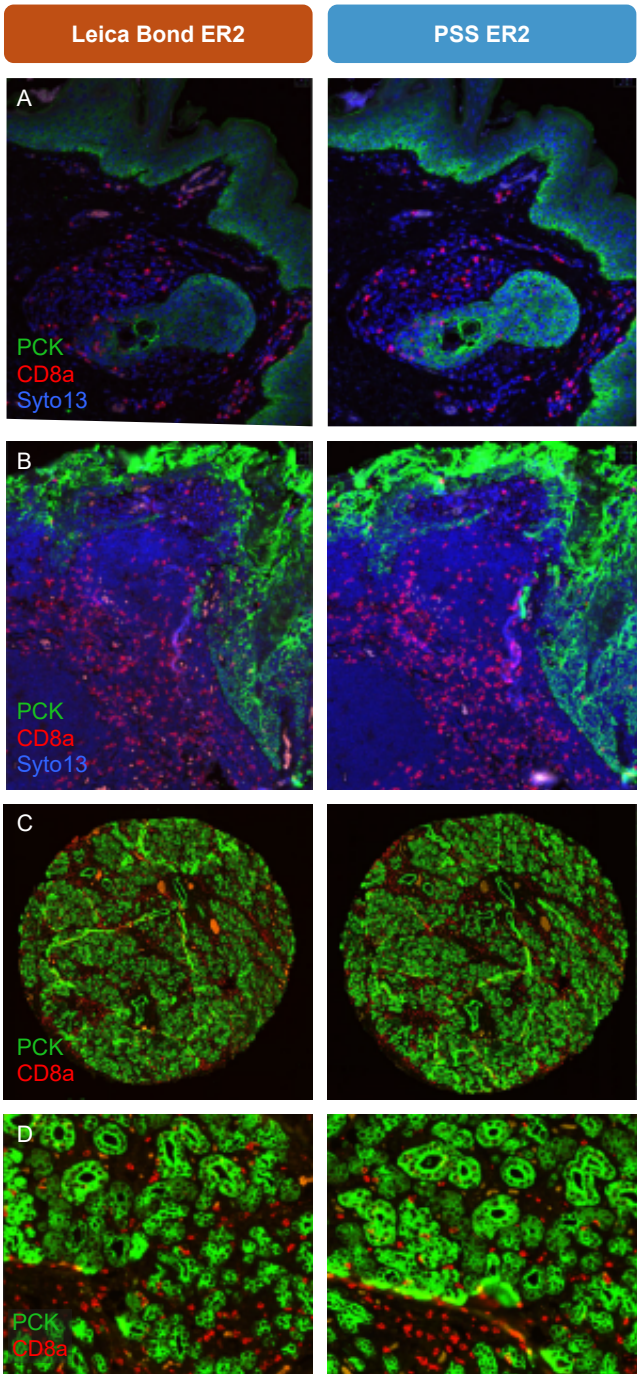


Figure 5: Comparison of Dewax+HIER automation platforms on various tissues. The PSS (left) or Leica Bond (right) was used to process (A) skin and (B) tonsil, with antibody staining colors indicated: Pan cytokeratin (PCK, green), CD8a (red), and nuclei (Syto13, blue), and (C) salivary gland. (D) A zoom-in salivary gland is also presented.

The PSS protocol yielded a stronger staining signal on skin tissue, a particularly challenging specimen type **(Figure 5A)**. Tonsillar tissue is commonly evaluated for spatial immune cell organization, and the automated PSS method again provided more robust staining of epithelial tissue and CD8+ T cells **(Figure 5B)**. These findings may be attributed to the superior dewaxing reagent and refined protocol.

The two automated methods processed several tissues in the microarray with comparable quality. **Figure 5C-D** presents examples of the salivary gland, both as an overview and in a zoomed-in view. Other tissues exhibiting similar staining profiles included the kidney, colon, spleen, and cervix (data not shown).

The dewax-plus-epitope retrieval processing of placenta **(Figure 6A-B)**, lung **(Figure 6C)**, and thyroid **(Figure 6D)** tissues on the Leica Bond automation platform yielded higher autofluorescence artifacts in the red channel for the CD8a antibody compared to the PSS platform. These experiments were conducted using the same ER2 epitope retrieval buffer and antibody stains; therefore, the reduced autofluorescence observed with the PSS is likely attributed to the quality of the dewax and antigen retrieval.

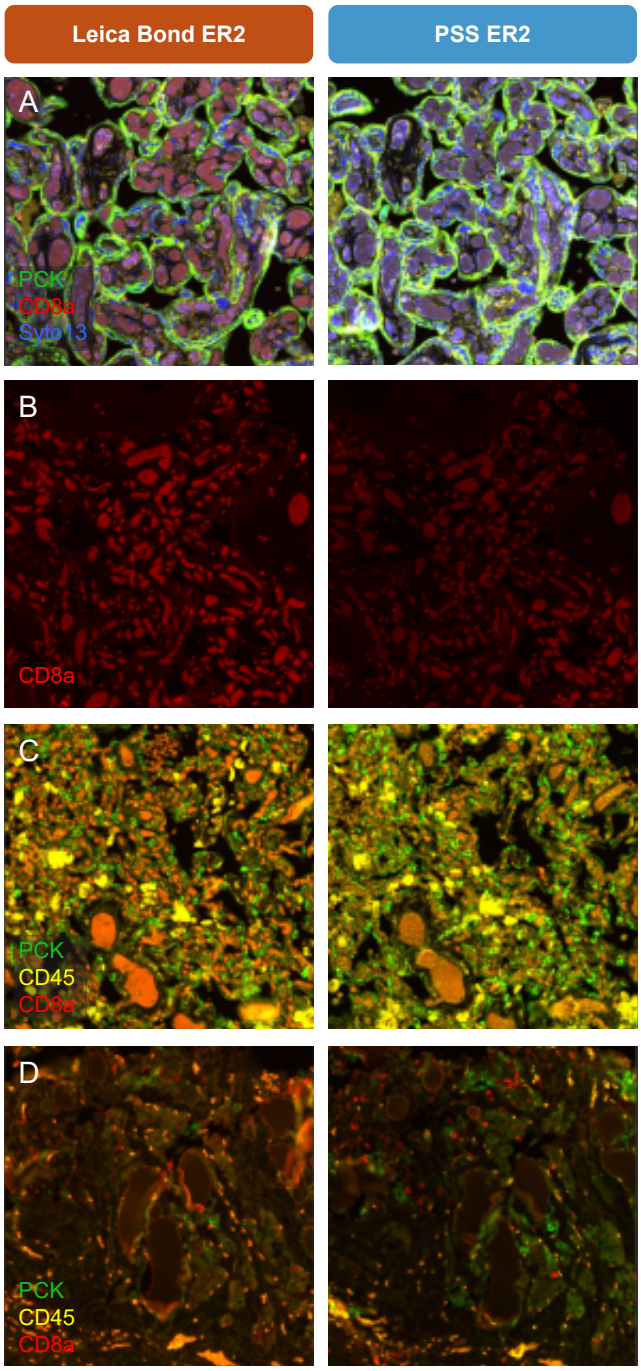


Figure 6: Human tissues exhibit increased autofluorescence in the red channel in Leica Bond (left) compared to PSS (right). (A) Placenta composite staining, (B) placenta with only CD8a visualized, (C) lung composite staining, and (D) thyroid composite staining.

Parhelia universal G-buffer rescues antigen retrieval for CD44

CD44 is a receptor for hyaluronic acid expressed on different cell types, including immune cells and cancer cells. Thus, CD44 is considered an important target for cell phenotyping and characterization of the tumor microenvironment. Despite being well recognized on fresh frozen mouse samples, CD44 detection is challenging in human FFPE samples, even when using multiple antibody clones. One possibility for the weak detection in human FFPE samples is that generalized antigen retrieval conditions are not optimal for CD44.

To simplify the choice of HIER alkaline buffer, Parhelia developed a universal solution (G-buffer) for its Skylab Dewax/HIER Kit. Using successive mesothelioma tissue samples processed automatically on the PSS (i.e., dewaxing, HIER, and staining), bulk G-buffer performed similarly to the other alkaline HIER buffers, ER2 and pH9 **(Figure 7A)**. When optimizing antigen retrieval conditions on the PSS, we paid special attention to CD44. In experiments performed, HIER with G-buffer yielded better CD44 staining contrast than pH9 or ER2 antigen retrieval solutions **(Figure 7B)**.

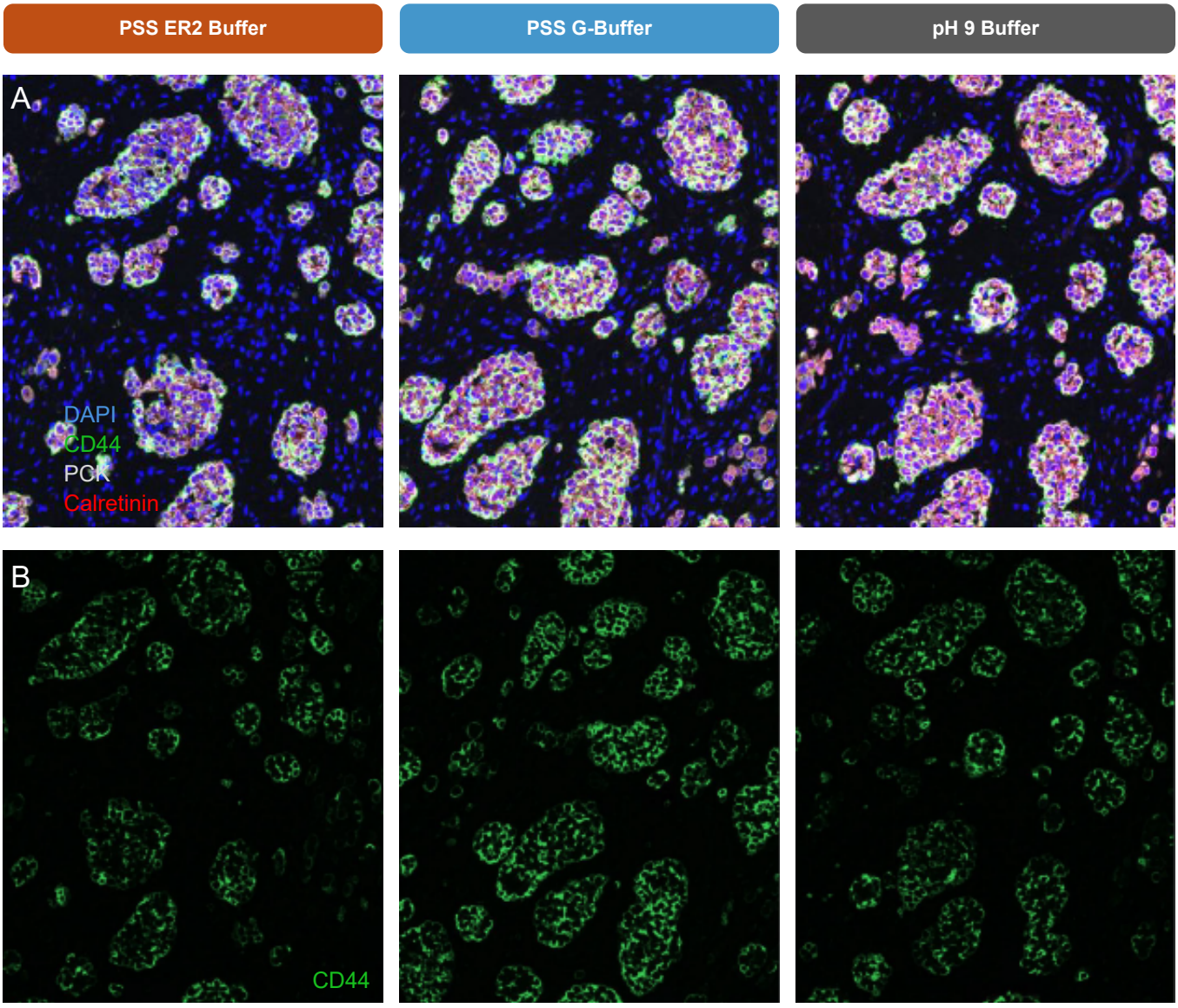


Figure 7: HIER alkaline buffer comparison of mesothelioma tissues prepared automatically on the PSS. (A) Composite images of tissues DAPI-stained nuclei (blue), CD44-antibody (green), PCK-antibody (light gray), and Calretinin-antibody (red). (B) CD44-specific staining. Note the stronger contrast for the middle specimen processed using G-buffer for the antigen retrieval step. Images were acquired on a PhenoCycler Fusion platform.

Skylab Dewax/HIER Kits represent a simple and consistent consumable for automating and scaling microscopy slide processing

Skylab Kits containing Parhelia Dewax and either HIER pH9 or G-buffer were produced in a single lot and distributed among labs in Maryland (NCI) and California (Stanford) for testing. At NCI, both Skylab Kit buffers performed similarly on sections from the mesothelioma specimens described in **Figure 7**, providing excellent contrast for all fluorescent markers, including CD44 **(Figure 8)**. As in the bulk buffer comparison, the G-buffer in the Skylab Kit yielded higher contrast for CD44 **(Figure 8B)**. Thus, the Skylab Dewax/HIER Kits are suitable for scaling the slide sample preparation for multiplex immunofluorescence microscopy studies.

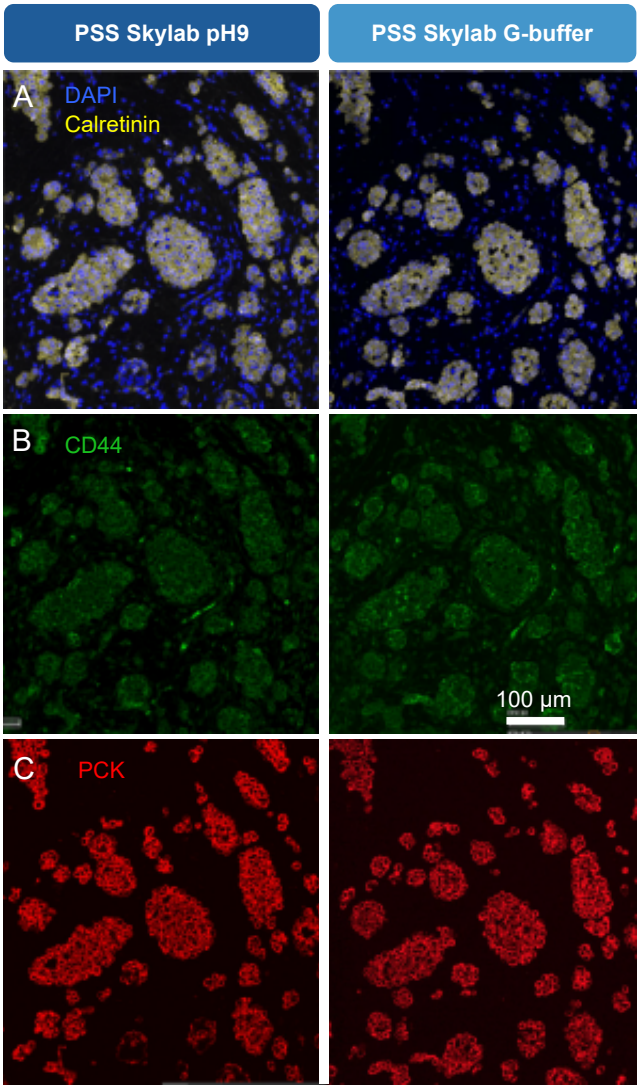


Figure 8: Testing of Skylab Dewax/HIER Kits for multiplex immunofluorescence applications. Staining of human mesothelioma tissue targeting (A) nuclei (DAPI, blue), Calretinin (yellow), (B) CD44 (green), (C) PCK (red). Images were acquired on a PhenoCycler Fusion platform.

At Stanford, immunohistochemistry (IHC) techniques were employed to assess Skylab Kit performance in chromogenic detection and to evaluate the workflow differences between manual and automated methods. Chromophore staining for the B cell marker CD20 resulted in similar quality among the generic pH9 HIER buffer for tonsil specimens processed manually with xylenes and the PSS-automated Skylab Kit **(Figure 9A)**. When applied to the leukocyte common antigen CD45, manual dewaxing and antigen retrieval using pH9 buffer also yielded similar staining quality comparable to automated processing and staining with G-buffer on the PSS **(Figure 9B)**. The Skylab Dewax/HIER Kit also exhibited minimal artifacts, while manual processing occasionally formed bubbles that resulted in unstained areas (discernible in the zoom-out insert, **Figure 9B**).

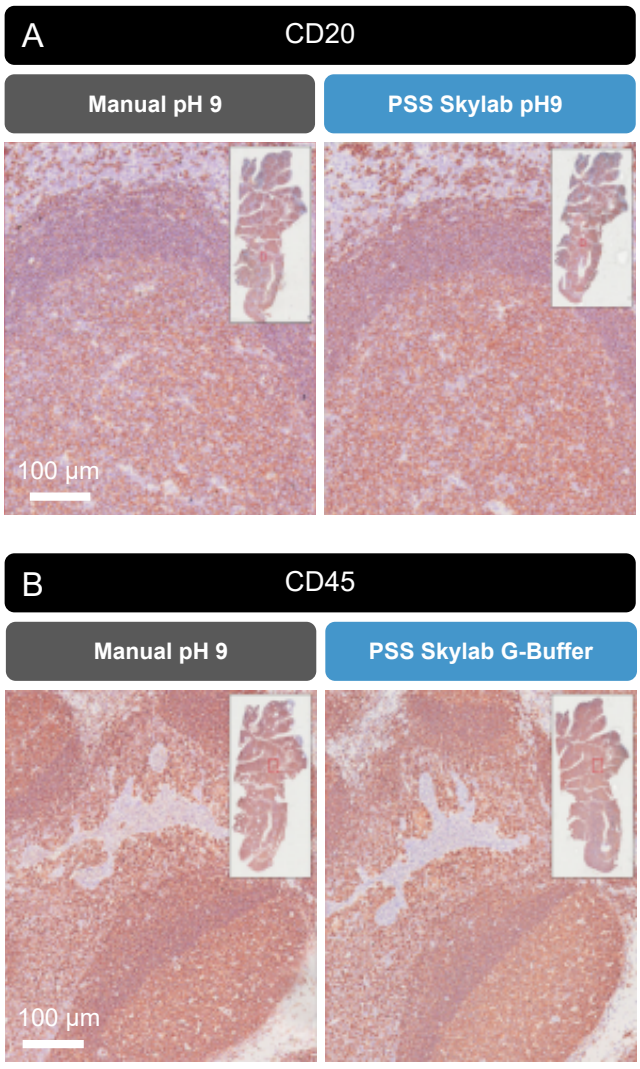


Figure 9: Testing of Skylab Dewax/HIER Kits for immunohistochemistry applications. Human tonsil samples were processed manually or automatically on the PSS using Skylab Dewax/HIER Kits. Chromogenic staining for (A) CD20 and (B) CD45. Images were acquired on a PhenoCycler Fusion platform.

Setting up one slide for end-to-end automated IHC slide processing on the PSS takes about five minutes of hands-on time to attach the CoverPad and prepare the run. In contrast, manual dewaxing and antigen retrieval require 10–15 minutes of hands-on time, totaling 20–30 minutes, depending on sample size and protocol details. Furthermore, the Skylab Kit includes all necessary reagents, providing 300 µL for each dewax and HIER step. This is about two orders of magnitude less reagent usage than manual processing, which requires 40–60 mL per step.

Overall, these results demonstrate that the Skylab Dewax/HIER Kit provides staining quality comparable to that of manual methods while significantly reducing operator hands-on time and reagent usage. Thus, the Skylab Dewax/HIER Kit represents a scalable approach to automating routine microscopy slide sample preparation protocols.

Skylab Dewax/H&E Kits deliver simplified and uniform staining of FFPE samples

We previously demonstrated automated hematoxylin and eosin staining using the PSS following multiplex proteomic phenotyping on the PhenoCycler Fusion.[9] Here, we extend that capability to Skylab Kits designed for H&E morphology staining. Several human tissues were dewaxed and stained with H&E using Skylab Kits, including lymph nodes **(Figure 10A-B)**, colorectal cancer liver metastasis **(Figure 10B)**, liver tumors, pancreatic tumors, and normal and inflamed thyroid glands (not shown).

The images in **Figure 10** demonstrate the uniformity of staining that results in the resolution of fine cellular morphological details. Each single-use kit, comprising eight reaction tubes and enclosed within the PSS, enables clean and consistent morphology staining, serving as a single diagnostic biomarker or complementing higher plex imaging techniques.

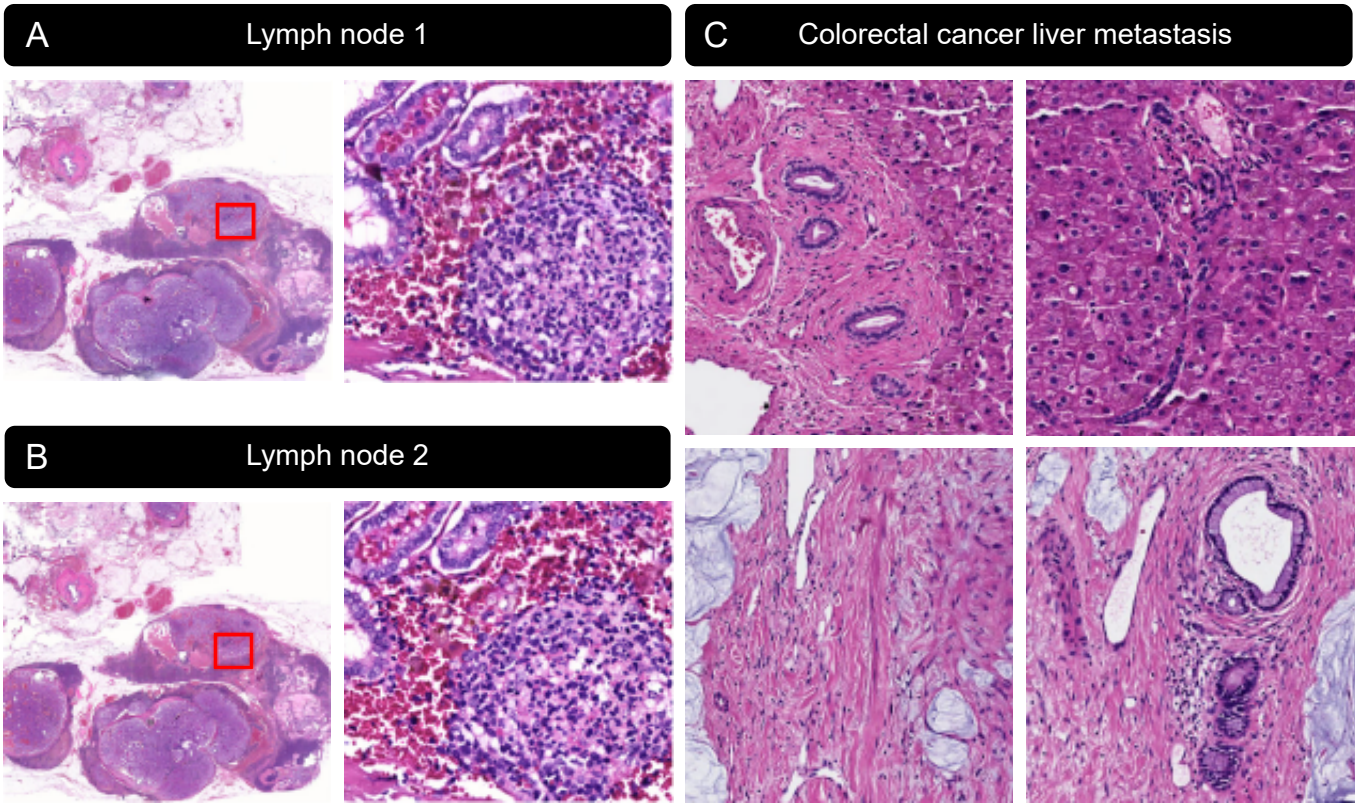


Figure 10: Skylab Dewax/H&E Kits enable uniform and scalable morphological staining in diverse tissues, including (A–B) two different lymph node sections and (C) four different regions of a colorectal cancer liver metastasis specimen. Red boxes denote zoomed-in sections, shown to the right in A–B.

Conclusions

In this report, we describe the automation of the deparaffinization and antigen retrieval protocols for FFPE-preserved tissue using the Parhelia Spatial Station. The dewaxing and HIER methods are comparable to manual processing and established automation on the Leica Bond, with added benefits of reduced hands-on time and reagent consumption, respectively. The PSS automated protocol can be widely applied to several tissue types, as demonstrated with a tissue microarray. Compared to automation on the Leica Bond, dewax and HIER using Parhelia reagents on the PSS often resulted in lower autofluorescence and better signal-to-noise ratio for multiple antibodies, providing evidence for improved reliability and reduced artifact generation. Combined with scalable, single-use Skylab Kits comprised of superior dewax and HIER reagents and verification of several spatial biology assays, these results signify the PSS as an end-to-end automation platform capable of standardizing spatial sample prep workflows while minimizing operator hands-on time and inter-operator variability.

Supplementary Table

Supplementary Table 1. Antibodies and fluorophores used in this study.

Antibody	Fluorophore
CD3 (rabbit, Abcam)	AF594 (donkey, anti-rabbit)
EPCAM (rabbit, CST)	AF594 (donkey, anti-rabbit)
CD44 (donkey)	AF647 (donkey, anti-rat)
CD8a (Novus)	AF647 (directly conjugated)
PCK	AF532 (directly conjugated)
Syto 13	AF488
Syto 83	AF532
E-cadherin	AF647 (directly conjugated)
CD45	AF594 (directly conjugated)
Calretinin	AF594 (directly conjugated)
Antibody	**Enzyme/substrate**
CD20	HRP/DAB
CD45	HRP/DAB

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