Walking a thin line between fixation and epitope binding – characterization of antigen retrieval methods suitable for eosinophil and HSV-2 staining in formalin-fixed female reproductive tissue

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ABSTRACT

Antibody-based fluorescence analysis of female reproductive tissues in research of sexually transmitted diseases allows for an in-depth understanding of protein localization, interactions, and pathogenesis. However, in many cases, cryosectioning is not compatible with biosafety regulations; at all times, exposure of lab personnel and the public to potentially harmful pathogens from biological infectious material must be avoided; thus, formaldehyde fixation is essential. Due to formaldehyde's cross-linking properties, protein detection with antibodies can be impeded. To allow effective epitope binding during immunofluorescence of formalin-fixed paraffin-embedded vaginal tissue, we investigated two antigen retrieval methods. We tested these methods regarding their suitability for automated image analysis, facilitating reproducible quantitative microscopic data acquisition in sexually-transmitted disease research. Heat-based retrieval at 80°C in citrate buffer proved to increase antibody binding to eosinophil protein and HSV-2 visibly and tissue morphology best, and was the most efficient for sample processing and quantitative analysis.

Key words: female reproductive tract tissue; fluorescence microscopy; immunoflourescence; immunohistochemistry; antigen retrieval; formalin-fixed and paraffin-embedded tissues; sexually transmitted diseases; Herpes simplex virus-2.

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Contributions: MR, KA, GK, AH, WH, designed and directed the project; LMW performed the animal experiments, analyzed images and developed the theoretical framework, drafted the original manuscript; LMW, ED, performed the immunofluorescence experiments and microscopy; MR, KA, critically assessed and amended the manuscript. All the authors read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

Conflict of interest: the authors declare they have no competing interests.

Ethics approval: all protocols and experiments were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Recklinghausen, Germany (81.02.04.2018.A341).

Funding: this work was supported by the German Research Foundation (DFG) within the German-African Projects in Infectiology (GSAT project; LA 2746/2-1). Moreover, AH is supported by the Federal Ministry of Education and Research (BMBF) [01KA1611, 01KA2027 and 01KA2113A] and additionally funded by the DFG under Germany's Excellence Strategy – EXC2151 – 390873048. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. This work was supported by the German Center for Infection Research (DZIF).

Introduction

Formalin-fixed, paraffin-embedded (FFPE) tissues have been the gold standard for diagnostics and research for many years, forming a significant collection of material worldwide.[1,2] Many laboratories have recognized the potential of using FFPE tissue sections for research and diagnostics of pathological disease abnormalities. One of the main benefits of formaldehyde fixation is that while preserving the tissues' structural integrity,[3] it inactivates infective organisms and viruses.[4] However, formaldehyde and other chemical fixatives change protein structures by cross-linking the residues of amino acids.[5] This can reduce, weaken, or inhibit sensitive antibody binding to antigen epitopes during immunohistochemical (IHC) analyses,[6] mainly for immunofluorescence (IF) but also for chromogenic evaluation of tissues. Antigen retrieval has been established for several species and tissues to break up such cross-links and allow effective antibody binding. Enzymatic or heat-based antigen retrieval methods depend on pH, incubation duration, and antigen target.[7,8] However, generally, not all tissue sections need to be treated, depending on target identity, antibody, tissue type, and fixation method and duration.[5,9,10] Here, we show the comparison of two antigen retrieval methods for IF histochemistry of the murine female reproductive tract (FRT) for two antigens apart from DAPI (4,6-diamidino-2-phenylindol), which has only been described by few authors previously[11,12] and no standardization has been established.[13] These methods can be tested in diagnostics and basic research regarding sexually-transmitted diseases of the FRT.

Immunohistochemistry of tissues can visualize protein location and interactions, painting a clear picture of cellular migration behavior. To demonstrate the method's great potential, we show an automated quantitative analysis of eosinophil counts of IF-stained vaginal and lung tissue from HSV-2-infected mice.

Materials and Methods

Animals

Female BALB/c mice were obtained from Janvier Labs (Le Genest-Saint-Isle, France) and kept at the animal house of the Institute for Medical Microbiology, Immunology and Parasitology (IMMIP), University Hospital Bonn (UKB), under specific-pathogen-free (SPF) conditions according to the German animal protection laws and EU guidelines 2010/63/E4. The mice had access to food and water *ad libitum*. All protocols and experiments were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Recklinghausen, Germany (81.02.04.2018.A341).

Sample source

Mice (8-12 weeks old) were infected naturally with the murine filaria *Litomosoides sigmodontis*[14,15] and subsequently co-infected with Herpes Simplex Virus 2 (HSV-2; $5x10^5$ PFU intravaginally, strain G, ATCC VR-734) for 7 days.[16] To facilitate HSV-2 infection and synchronize the estrous cycle, progesterone (2 mg/19 g body weight) (Depo-Provera®, Pfizer, New York, NY, USA) was administered subcutaneously 7 days prior to HSV-2 infection. The results shown here are representative of three experiments, each with at least four mice.

Tissue preparation for histological analysis

FRT and lung tissue were obtained and organs were placed into a tissue embedding cassette (Carl Roth, Karlsruhe, Germany), fixed in 4% formaldehyde (Carl Roth) for 24 h, and then stored in distilled water at room temperature (RT). Dehydration was performed after the fixation using a Spin Tissue Processor STP-120 (Especialidades Médicas MYR S.L., Tarragona, Spain). Dehydration was achieved by immersing the tissue in ascending grades of ethanol (Merck, Darmstadt, Germany). Afterward, the alcohol was replaced with xylene (Engelbrecht, Munich, Germany) and then with paraffin. Before the vertical embedding of the vagina, the distal part of the FRT was separated from the ovaries and the uteri, which were embedded horizontally. The lungs were embedded with the ventral side down horizontally. The embedding was performed with the HistoServe tissue embedding station EC350 (Especialidades Médicas MYR S.L). Finally, 4-5 µm thin sections were cut using the Rotary 3005E semi-electronic rotary microtome (PFM Medical, Cologne, Germany). The tissue sections were transferred onto slides (SuperFrost™ Plus, ThermoFisher Scientific, Waltham, MA, USA), incubated at 37°C for 24 h and then stored at 4°C until staining.

Antigen retrieval and immunohistochemistry

Optimal staining protocols have not been described yet for murine FRT tissue, which is why we compared two commonly known and well-established AR methods to each other and non-treated sections. Proteolytic-induced epitope retrieval (PIER) with Proteinase K and heat-induced epitope retrieval (HIER) in citrate buffer were compared to revert formaldehyde-induced changes in epitope conformation and break cross-linking. Additionally, lung FFPE tissues were also treated and stained for comparison.

Proteinase K antigen retrieval

Enzymatic antigen retrieval is based on the proteolytic breaking of cross-links in antigenic molecules.[17] Thus, we tested the commonly available and in molecular laboratories frequently used enzyme Proteinase K. Following deparaffinization and hydration, Proteinase K (Qiagen, Hilden, Germany) working solution (0.6 units/mL, as described by https://www.ihcworld.com/_protocols/epitope_retrieval/proteinase-k.htm) was pipetted onto the slides. The sections were placed into a humidity chamber (Biozol, Eching, Germany) and incubated for 15 min at 37°C; then, a cooling-off period of 10 min at room temperature followed in tris buffered saline (TBS).

Heat-based antigen retrieval in citrate buffer

Heat-based antigen retrieval methods have been proven effective previously;[8,18] however, no standardized protocol has been established for vaginal tissue. Sections were placed into the Epredia™ Lab Vision™ PT module (ThermoFisher Scientific) filled with citrate buffer (1:100, Vector laboratories, Newark, CA, USA) at 65°C. After heating up to 80°C, sections were incubated at that temperature for 20 min. A cooling-off period of 15-25 min followed.

Immunohistochemistry of FRT tissue

The tissue sections were blocked (10% donkey serum (VWR, Darmstadt, Germany) and then washed with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MI, USA) in TBS for 30 min. After washing, an incubation with the primary antibody followed at 4°C overnight. Rabbit-anti-RNASE3 (eosinophilic cationic protein; ECP) (1:250, SA5-10039, ThermoFisher Scientific) and sheep-anti-HSV-2 (1:250, ABIN473182, antibodies-online.com) were used as primary antibodies. The next day, the antibody solution was tapped off carefully and the slides were washed in TBS for 5 min. The secondary antibody (donkey-anti-rabbit DyLight550, SA510039, Thermo Fisher Scientific, donkey-anti-

sheep Alexa Fluor 647 (A21448, ThermoFisher Scientific) was diluted 1:250 in 0.1% bovine serum albumin (PAA Laboratories, Pasching, Austria) in TBS for 60 min. A quenching solution (Vector TrueVIEW Autofluorescence Quenching Kit, Biozol) was pipetted onto the tissue samples, to minimize the background signal due to the autofluorescence of the tissue. Vectashield® anti-fade mounting medium with DAPI (Vector laboratories) was used for cover-slipping (24x50 mm, 170 µm, Marienfeld, Lauda-Königshofen, Germany). Negative controls were performed to ensure the specificity of IF signals. Therefore, slides of the same samples were incubated with 0.1% bovine serum albumin in TBS without primary antibodies parallel to the other samples; the same secondary antibody concentrations as for the other samples were applied.

Microscopic analysis

Microscopic evaluation, photography, and image processing were performed with a Zeiss Axio SlideScan Z.1 (Zeiss, Oberkochen, Germany) and the Zen Software 2.6 or Zen lite 3.5 (Zeiss) and the objective Plan- Apochromat 20x/0.8 M27, was used with the Orca Flash 4.0 V3 camera (Hamamatsu, Shizuoka, Japan). Exposure times of 2.07s, 601.3 ms, and 24 ms for Alexa Fluor 647, DyLight 550, and DAPI, respectively, were applied for all images equally to ensure comparability. In addition, image brightness editing was also applied equally for all sections. For automated image evaluation, the Zeiss Zen Image Analysis Software Add-On was used. Here, automatic segmentation for a region of interest (ROI) of 2000x1600 pixels was determined. The following settings were defined for the DAPI and 555 nm channel equally. Gaussian smoothing method and edge enhancement were chosen according to signal intensity and the threshold was adjusted and defined with the histogram. For optimal separation, the watershed method was applied. The size of the object was filtered with a minimum and maximum, after that a manual correction of software segmentation errors was performed blinded, such as separation of clustered signals, erasing of artifacts, and drawing of unrecognized events. Eosinophil counts were divided by nuclei counts of the ROI.

Results

To improve fluorescence images and automated image analysis of ECP in murine FRT sections, the following antigen retrieval methods were tested: HIER with citrate buffer and PIER with Proteinase K. These methods were evaluated regarding not only the improvements of the tissue and immunoreactivity itself but also the feasibility of performing such a method with many samples in a short amount of time.

Processing time

Preparation and tissue processing for HIER with citrate buffer was by far the easiest and quickest method, taking about 35 min. Pipetting the Proteinase K solution on the individual slides, incubation, and cooling off took all together as long as the entire citrate buffer treatment and cooling off.

Tissue and signal quality

Tissues were evaluated regarding structural integrity, fluorescence properties, and morphological details (Figures 1 and 2). For immunological studies, it is vital to analyze morphological details, cell-cell and cell-pathogen interactions. Thus, close-up images were used to assess which antigen retrieval is the most suitable technique (Figure 2). Figures 1 and 2 show the tissues from the

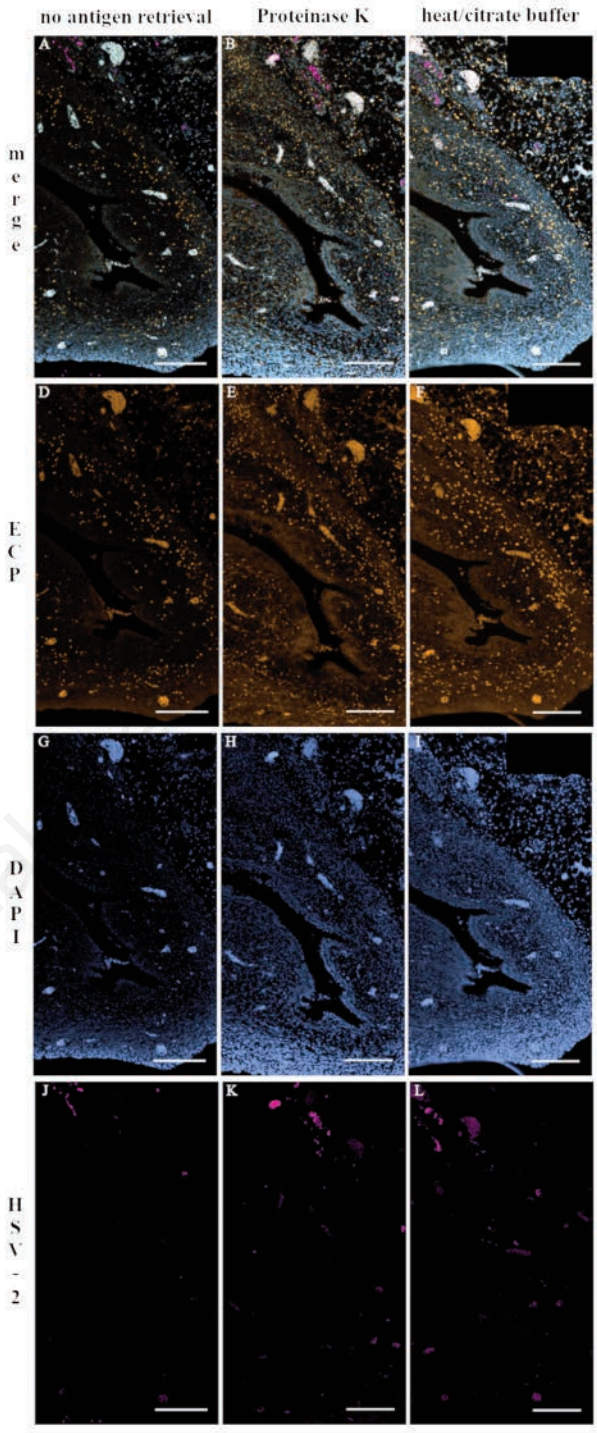

Figure 1. Microscopic images of vaginal tissue section after antigen retrieval treatment demonstrate improved tissue morphology and antibody binding due to HIER. Left to right: no treatment (**A**) of vaginal tissue shows blurry tissue-structures and weak DAPI and ECP signals. Proteinase K (**B**) proteolytic treatment of vaginal tissue reduced background signal, improved structural morphology, and ECP and DAPI signal. Citrate buffer-heat treated (**C**) vaginal tissue improved signal intensity visibly the most; background autofluorescence and structural blurriness were reduced strongly. Top to bottom: Merge of all channels, 555 nm wavelength showing ECP (orange) localization, DAPI (blue) detecting DNA localization with emission wavelength of 465 nm, 633 nm showing localization of HSV-2 (pink) infected-cell-proteins (ICPs). The tissue sample itself and image ROI are all identical among the images. Scale bars: 200 µm.

same sample without antigen retrieval (Figures 1 and 2 A,D,G,J), enzymatic treatment with Proteinase K (Figures 1 and 2 B,E,H,K), and heat treatment in citrate buffer (Figures 1 and 2 C,F,I,L). ECP is shown in orange and was detected at 555 nm, DAPI (blue) shows DNA localization at 465 nm, and HSV-2 infected cell proteins (ICPs) are shown in purple, detected at 633 nm. The tissue structure of untreated (Figures 1-2 A) samples appeared the most uneven and blurry. These samples had the least clear nuclear and cellular structures (DAPI signal) (Figures 1 and 2 G). Especially the epithelial and stromal cells of vaginal tissue showed blurriness, background autofluorescence, and faint ECP and DAPI signal, if untreated. Proteinase K-treated samples had improved morphological details; however, the fluorescence signal was not as strong as with citrate buffer (Table 1). In the 633 channel, this treatment seemed to increase background autofluorescence slightly (Figure 1 and 2 K). Overall, the heat/citrate buffer-treated tissues showed the strongest fluorescence signal in all channels (Figure 1 and 2 C). During quantitative analysis, DAPI segmentation was achieved most accurately with heat/citrate buffer treatment. For untreated (Figure 3A) vaginal samples, the software was not able to distinguish between DAPI-positive nuclei due to weak signal and strong background autofluorescence. Thus, normalization of eosinophil counts to all cells in the ROI was not feasible. Either the software counted very few signals when several nuclei and background were merged into one segment (Figure 3A, asterisk) or it divided one nucleus into several signals, creating more signals than nuclei present (Figure 3A, arrow; Table 2). Upon manual control of image analysis segments, nuclei counts were most accurate for heat-treated samples and had only a few, if any incorrectly segmented nuclei. Thus, in Table 2, values of heat-treated tissues can be used as a reference for accurate eosinophil count numbers. Analysis of Proteinase K-treated tissues had minor errors in DAPI segmentation; however, it also produced reliable cell counts. Interestingly, lung tissues (Figure 4) showed marginal differences in fluorescence signal between the antigen retrieval methods and untreated tissues (Figure 4A, Table 3). Morphological details of nuclear staining with DAPI appeared best in Proteinase K-treated lungs (Figure 4 B,H). Heat/citrate buffer treatment improved signal intensity in the 555 channel (Figure 4 C,F) but did not improve DAPI intensity or tissue morphology (Figure 4 C,I) compared to Proteinase K treatment. In summary, HIER treatment of FRT tissue sections was the most efficient method to improve tissue structure, antibody binding (fluorescence signals), and thus automated image analysis of ECP. Lung tissue sections treated with PIER also showed increased fluorescence signal and tissue structure compared to untreated tissues.

Discussion

Undoubtedly, molecular-based analysis of FFPE tissues is a vital tool for basic research, diagnostics and drug development. FFPE tissues make up the majority of medical and biological samples in diagnostic and research laboratories. However, worldwide standardization of IF staining or antigen retrieval has never been accomplished successfully.[19] This methodological paper aimed to demonstrate the potential that FFPE vaginal tissue samples have for research of infectious diseases if processed properly. Due to the epitope cross-linking and DNA degradation by formalin fixation, the fixative decreases the antibody-binding properties of antigens.[5,6] To enable microscopic detection of fluorescent antibodies, antigen retrieval is a common method of immunohistochemistry protocols. However, tissues and antigens differ in their autofluorescence and susceptibility to cross-linking of formaldehyde.[5,9,10] We tested two antigen retrieval methods for IF of vaginal tissue to

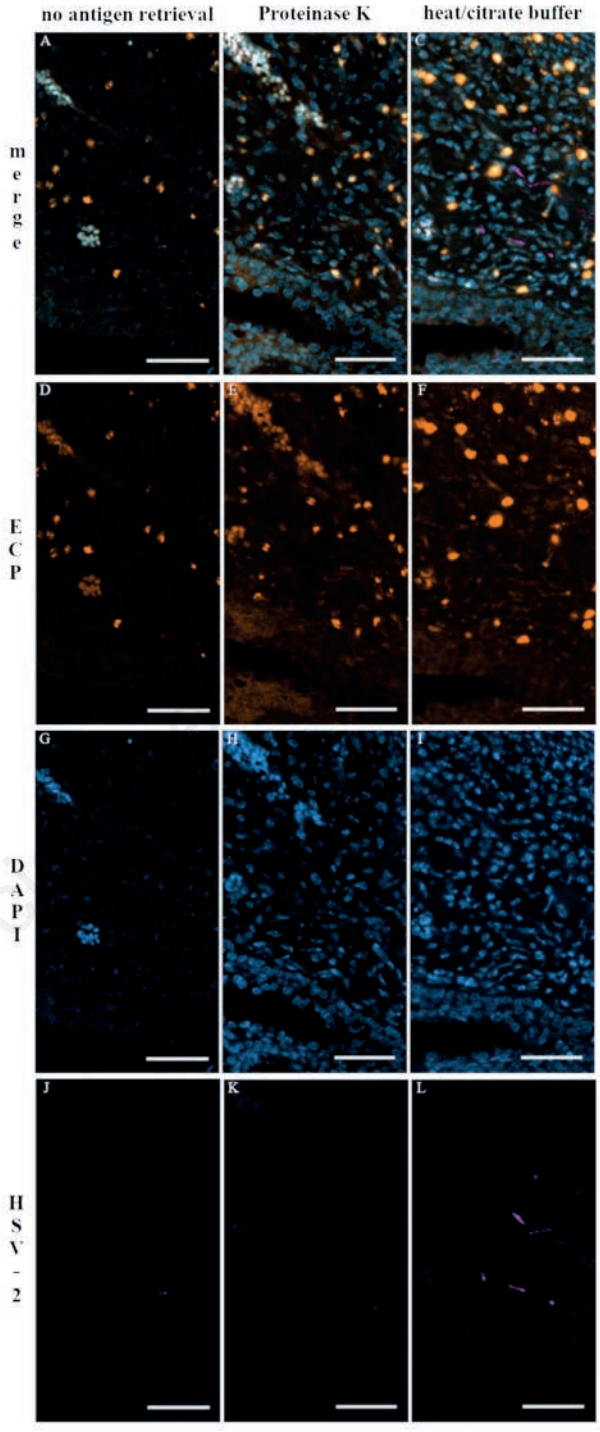

Figure 2. Close-up microscopic image of antigen retrieval treated and untreated FRT tissue samples. Left to right: no treatment (**A**) of vaginal tissue shows blurry tissue structures and weak DAPI and ECP signals. Proteinase K (**B**) proteolytic treatment of vaginal tissue reduced background DAPI signal, improved structural morphology, and ECP and DAPI signal; however, tissue autofluorescence in the 633 nm channel appeared increased slightly. Citrate buffer-heat treated (**C**) vaginal tissue improved signal intensity visibly the most; background autofluorescence and structural blurriness were reduced strongly. Top to bottom: Merge of all channels, 555 nm wavelength showing ECP (orange) localization, DAPI (blue) detecting DNA localization with emission wavelength of 465 nm, 633 nm showing localization of HSV-2 (pink) ICPs. The tissue sample itself and image ROI are all identical among the images. Scale bars: 50 µm.

optimize a protocol for FFPE FRT eosinophil, HSV-2 and nucleus staining with minimal variability for automated quantitative analysis. This method can then be used to understand immune cell compositions and dynamics in the vaginal tract during infections. Pudney *et al*.[12] have successfully used antigen retrieval by heat for vaginal biopsies to analyze and quantify human immune cell composition with IHC; however, they performed chromogenic IHC of human tissue, whereas we demonstrate and compare antigen retrieval methods for IF staining of murine tissues.

In our experimental setup, HIER antigen retrieval at 80°C in citrate buffer with a pH of 6.0 proved to be the most effective treatment of tissue slides regarding the fluorescence intensity of our chosen antibodies, structural integrity, and morphological details, confirming previous publications showing that these conditions are suitable for paraffin-embedded tissues.[18,20] Reagent preparation and procedure were quick and easy to perform, making this protocol optimal for FRT tissue of eosinophil and HSV-2 IF staining, especially if strong fluorescence intensity is desired and a high sample load needs to be processed standardized, for example in a clinical trial.

Enzymatic Proteinase K antigen retrieval also improved signal intensity, especially in the lung tissue, and reduced blurriness of the tissue, so this method is recommended if reagents or machines are not available or for a low sample load. Since other publications have warned of non-specific cleavage and uncontrollable enzymatic reactions - potentially leading to a negative, unwanted proteolytic effect on antigens[18,21] - we did not incubate the tissue longer than 15 min. Thus, it cannot be excluded, that longer incubation times might improve immunoreactivity.

Compared to the vaginal tissue, the Proteinase K and HIER antigen retrieval of the lung tissue only had little differences in signal intensity and morphological integrity, showing how IF and antigen retrieval can vary depending on the organ. Additionally, antigen retrieval methods and specific parameters like pH, temperature, and incubation duration have to be individually tested for each antigen.[22] Thus, our findings are not necessarily applicable for other targets and tissues and further antigen retrieval methods need to be tested to obtain optimal laboratory-specific results. Based on our findings, we assume that other antigen retrieval methods will not lead to significantly increased ECP detection to improve automated microscopic analysis, as HIER antigen retrieval was sufficient and easily attainable for our purposes. Thus, additional experiments with higher sample counts, various antigens, and other retrieval methods could not be performed to fulfill the 3R (replacement, refinement and reduction) principle of animal welfare guidelines, which demands a reduction of mouse numbers and does not allow additional animal infection experiments for this purpose.

During microscopic analysis, exposure time and LED intensity were set accordingly for the untreated tissue and based on that, the other samples were imaged for comparability. Thus, the ECP sig-

Table 1. Antigen retrieval microscopic evaluation of compared methods for the FRT: Proteinase K and heat-treated tissue.

	Untreated	Proteinase K	Citrate buffer/heat
Tissue morphology	-	+	++
Autofluorescence	++	+	-
DNA signal	-	+	++
ECP signal	-	++	++
HSV-2 signal	+	+	+
Treatment duration	0 min	30 min (plus pipetting)	30 min

-, negative/weak; +, positive/strong; ++ very positive/strong.

Table 2. Image analysis results of 2000x1600 pixels ROI of the vagina tissue section. Compared are antigen retrieval untreated and treated results for ECP (eosinophil) and nuclei (DAPI signal) counts. Values were normalized (eosinophils/all nuclei of ROI). Heat-treated tissues were optimal for segmentation, indicating the inaccuracy of untreated numbers for nuclei.

	Untreated	Proteinase K	Citrate buffer/heat
ECP+ eosinophil	242	270	207
Nuclei (DAPI)	113	3318	2804
Eosinophils/all nuclei	2.14	0.08	0.07

Table 3. Antigen retrieval microscopic evaluation of compared methods for the lung: Untreated, Proteinase K and heat-treated tissue.

	Untreated	Proteinase K	Citrate buffer/heat
Tissue morphology	-	+	+
Autofluorescence	+	+	-
DNA signal	-	++	++
ECP signal	-	++	++
Treatment duration	0 min	30 min (plus pipetting)	30 min

-, negative/weak; +, positive/strong; ++ very positive/strong.

nal was over-exposed in the heat/citrate buffer and Proteinase K-treated samples, due to improved antibody binding and weaker background autofluorescence signal. A reduced LED intensity and exposure time reduce the bleaching of antibody fluorophores. Therefore, heat-based antigen retrieval of FRT FFPE tissue in citrate buffer is desired, if the samples need to be analyzed for long periods of time or repeatedly to avoid bleaching.

This increased fluorescence signal after citrate buffer heat treatment was optimal for automated image analysis of ECP, as more signals were recognized by the software during segmentation with minimal variability and less false-positive counting occurred. This shows how improper tissue handling and microscopy can lead to false results and subsequently to incorrect quantitative data, as it is a problem in many publications.[9] Thus, optimized heat-/citrate buffer-based antigen retrieval treatment of vaginal tissues could improve diagnostics and research comparability of sexually transmitted infections and other antibody-based analyses of vaginal tissue samples, for example, microbiome or cancer research.

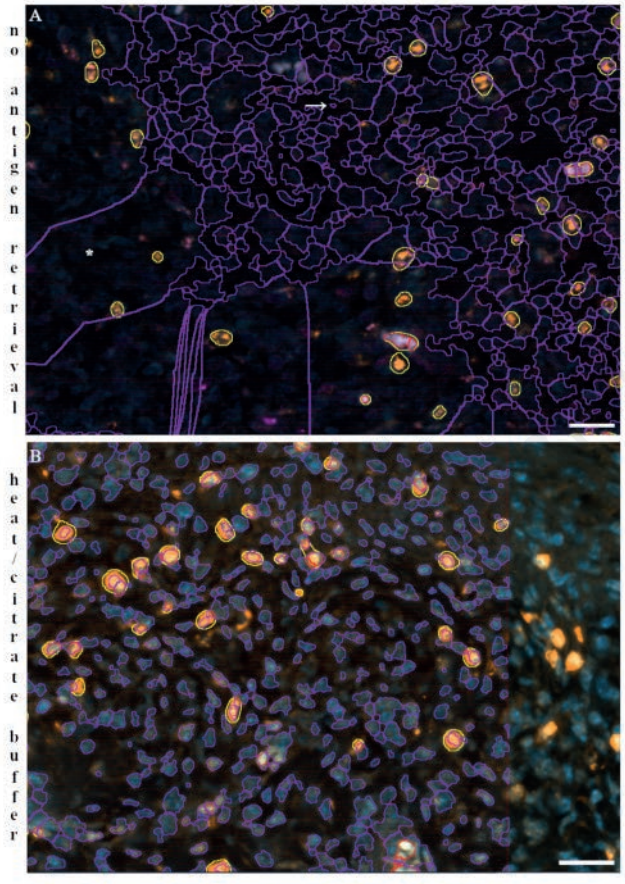

Figure 3. Accurate segmentation during image analysis of HIER treated vaginal tissue sections. The classes were colored in yellow, red, and pink. In the yellow regions 555 signal (ECP localization) was detected, red regions indicate a merge of DAPI and 555 signal, and pink regions show DAPI localization. **A**) Untreated tissue sections had such a low threshold of signal, leading to large segments if several nuclei were identified as one (asterisk as example), thus nuclei remained undetected. DAPI signals were divided into several counts in some instances (arrow as example). **B**) Heat-treated tissue sections allowed for correct segmentation and therefore accurate cell counts. ROI of analysis was performed on the same region in tissue sections of the same organ. Scale bars: 20 µm.

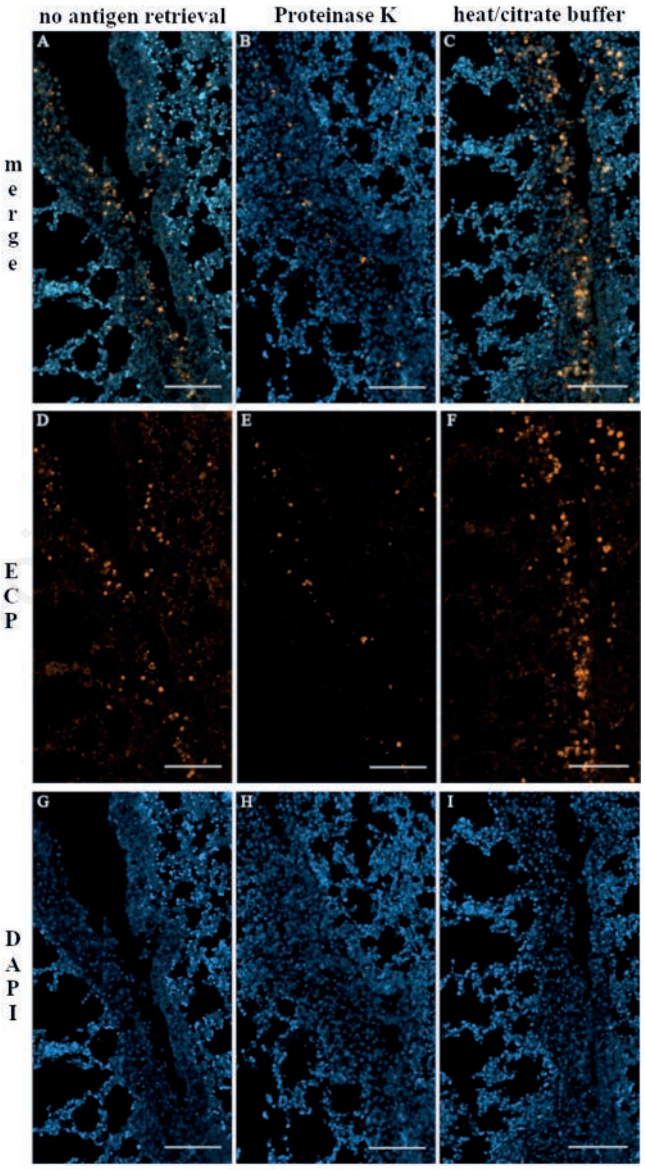

Figure 4. Microscopy images of antigen retrieval untreated and treated lung tissue samples show marginal differences in fluorescence signal intensity. Left to right: no treatment (**A**), Proteinase K (**B**), heat-/citrate buffer-treated (**C**). Top to bottom: Merge of all channels, 555 nm wavelength showing ECP (orange) localization, DAPI (blue) detecting DNA localization with emission wavelength of 465 nm. Proteinase K and heat/citrate buffer-treated lung tissues have less background autofluorescence, clearer and sharper morphology, and stronger fluorescence signal in both channels. The tissue sample and image ROI are all identical among the images. Scale bars: 100 µm.

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Received: 29 November 2023. Accepted: 19 March 2024.

European Journal of Histochemistry 2024; 68:3929
doi:10.4081/ejh.2024.3929