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Comparison of different methodologies and cryostat versus paraffin sections for chromogenic immunohistochemistry

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ABSTRACT

Immunohistochemistry (IHC) specifically localizes proteins in cells and tissues, but methodologies vary widely. Therefore, we performed a methodological IHC optimization and validation study. First, we compared advantages and disadvantages of cryostat sections versus paraffin sections. Second, we compared and optimized antigen retrieval in paraffin sections using citrate buffer and Tris/EDTA buffer. Third, aminoethyl carbazole (AEC) and 3,3'-diaminobenzidine (DAB) were tested as horseradish peroxidase (HRP) substrates to obtain a water-insoluble coloured end product to visualize antigens. Fourth, secondary antibodies conjugated with either mono-HRP or poly-HRP were compared. The study was performed using serial sections of human tonsil. IHC was performed with primary antibodies against endothelial cell marker CD31, smooth muscle actin (SMA), chemokine stromal-derived factor-1α (SDF-1α) and its receptor C-X-C receptor type 4 (CXCR4), macrophage marker CD68 and proliferation marker Ki67. DAB rather than AEC, and cryostat sections rather than paraffin sections gave optimum staining at highest primary antibody dilutions, whereas tissue morphology in paraffin sections was superior. Loss of antigenicity in paraffin sections by formaldehyde fixation, heat and/or masking of epitopes was counteracted by antigen retrieval but not for all antigens. Two out of six antigens (CD31 and CD68) could not be retrieved irrespective time and type of retrieval. Tris-EDTA was superior to citrate buffer for antigen retrieval. The use of mono-HRP or poly-HRP depended on the affinity of the primary antibody for its antigen. We conclude that IHC methodology optimization and validation are crucial steps for each antibody and each research question.

1. Introduction

Immunohistochemistry (IHC) is widely used to localize specifically proteins in cells and tissues (Matos et al., 2010; Nanduri and Prabhakar, 2018). Different chromogens are used for IHC such as aminoethyl carbazole (AEC) (Hira et al., 2015, 2018; Shimizu et al., 2017) and 3,3'-diaminobenzidine (DAB) (Breznik et al., 2018; Nanduri and Prabhakar, 2018) which are converted into red and brown water-insoluble end products, respectively, by the enzyme horseradish peroxidase (HRP) for light microscopical analysis of the proteins of interest in cells and tissues (Chen et al., 2010; Kumar et al., 2015). Furthermore, cryostat tissue sections are used (Hira et al., 2015, 2018) or paraffin sections after antigen retrieval (Breznik et al., 2018; Tani et al., 1992). However, as far as we know, only a few studies decades ago have been published comparing the use of AEC and DAB as chromogens (De Jong et al., 1985; Trojanowski et al., 1983) whereas a comparative study on the use of cryostat sections or paraffin sections after antigen retrieval has not been published yet.

Therefore, we aimed to perform a comparative chromogenic IHC methodological optimization and validation study using human tonsil to compare the advantages and disadvantages of serial cryostat versus paraffin sections, AEC versus DAB as chromogens and secondary antibodies conjugated with mono-HRP versus poly-HRP. We also compared the results of IHC on cryostat sections versus paraffin sections after various antigen retrieval methods (citrate buffer (pH 6.0) or Tris/EDTA buffer (pH 9.0) at 98 °C in a heating module for 10–40 min). We stained sections for 6 markers that are abundantly expressed in human tonsils, endothelial cell marker CD31 (Jackson et al., 2000), smooth muscle cell

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marker smooth muscle actin (SMA) (Honma et al., 1995), chemokine stromal-derived factor-1α (SDF-1α) and its receptor C-X-C receptor type 4 (CXCR4) (Corcione et al., 2000; Ehlin-Henriksson et al., 2006), macrophage marker CD68 (Jayakumar et al., 2005) and proliferation marker Ki67 (Hsu et al., 2013).

2. Materials and methods

2.1. Tonsil samples

Frozen human tonsil samples and paraffin-embedded tonsil samples of donors were obtained from the Amsterdam UMC, Pathology Department of the Academic Medical Center, Amsterdam, The Netherlands by Gabrielle Krebbers. Tonsil samples were obtained on the indication of a tonsillectomy and were stored anonymously as waste material. Therefore, approval of their use by the Ethics Committee was not required by Dutch law.

2.2. Chromogenic IHC using cryostat sections and paraffin sections of human tonsils

Chromogenic IHC experiments were performed on serial cryostat sections and paraffin sections of human tonsils. We stained for CD31 (PECAM1), SMA, SDF-1α (CXCL12) and its receptor CXCR4, CD68 (GP110) and Ki67. We compared AEC and DAB for their use as chromogens for IHC. Besides, we compared 2 types of secondary-HRP antibodies: mono-HRP versus poly-HRP; the latter is used for amplification of the signal.

2.3. IHC using human tonsil cryostat sections

Cryostat sections (7–μm thick) were cut at −25 °C on an Cryostar NX70 cryostat (Thermo Fisher Scientific, Waltham, MA, USA), picked up on glass slides, and stored at −80 °C until used. The final comparison of all variations in staining procedures including controls were performed on serial sections of one tonsil sample to allow direct comparison of the effects of variation of the staining procedures.

Cryostat sections were air dried at room temp for 15 min before immunohistochemical staining. The sections were fixed in acetone (−20 °C) (Lot nr. K49845114804; Merck, Darmstad, Germany) for 10 min and were air dried afterwards for 15 min followed by 3 washing steps of 5 min with Tris-buffered saline (TBS; pH = 7.6). The sections were treated with 100% methanol (Lot nr. I874509713; Merck) containing 0.3% H_2O_2 (Lot nr. K40899387013; Merck) for 10 min to block endogenous peroxidase activity to reduce non-specific background staining, followed by 3 washing steps of 5 min each using TBS containing 0.1% Triton-X (Lot nr. 072K0161; Sigma-Aldrich, St. Louis MI, USA) for permeabilization of the tissue sections. Sections were encircled with a PAP pen (Dako, Glostrup, Denmark) and incubated with TBS containing 3% normal goat serum (Lot nr. X0907; Dako) and 0.1% Triton-X for 1 h to further reduce non-specific background staining. Sections were subsequently incubated overnight at 4 °C with primary antibodies diluted in Normal Antibody Diluent (Ref nr. BD09-999; Lot nr. 150713; Immunologic, Duiven, The Netherlands) as indicated in Table 1. The 6 primary antibodies were first tested in a dilution of 1:200 with AEC and DAB as chromogens and subsequently the optimum dilution up to 1:2000 of the primary antibodies was established.

Two types of goat anti-mouse or goat anti-rabbit secondary HRP antibodies were tested: mono-HRP (Ref nrs. P0447, P0448; Dako) and poly-HRP (Ref nrs. VWRKOPVM55HRP, DPVR55HRP; Immunologic) followed by 3 washing steps of 5 min each using TBS. Next, sections were incubated with either AEC (peroxidase substrate kit; Cat nr. SK-4200; Vector Laboratories, Burlington CA, USA) for 20 min, or DAB (diluted in imidazole-HCl buffer containing H_2O_2, pH 7.5; Ref nr. K3468; Lot nr. 10121633; Dako) for 10 min, followed by one washing step with running tap water to stop the peroxidase enzyme reaction. Sections were then incubated for 30 s in hematoxylin (Ref nr. HHS32-IL; Lot nr. SLBS4574; Sigma-Aldrich) for nuclear counterstaining. Sections were again placed in running tap water for 5 min and then in distilled water. All incubation steps were performed at room temp, except for the overnight incubations with primary antibodies that were performed at 4 °C. Finally, the sections that were stained with DAB were dehydrated by dipping in 70%, 96% and 100% ethanol (Lot nr. K47696783613; Merck) and 3 times dipping in xylene (Lot nr. 18B274008; VWR Chemicals, Atlanta GA, USA). Finally, sections were covered with the synthetic mountant Pertex (Ref nr. 00811-EV; Lot nr. 1717227; Histolab, Götenburg, Sweden). Sections that were stained with AEC were covered with glycerin/gelatin mounting medium (Lot nr. SLBV3965; Sigma-Aldrich).

Control incubations were performed either in the absence of the primary antibody or in the presence of rabbit serum or mouse serum in the same concentration as the primary antibody to determine the effect of the serum on non-specific background staining.

2.4. IHC using human tonsil paraffin sections

Paraffin sections (5 μm thick) of human tonsil were stored at room temp until use. Dewaxing was performed by incubation of the sections in xylene (VWR Chemicals) for 10 min and rinsing in 100% ethanol (Merck). The sections were treated with 100% methanol (Merck) containing 0.3% H_2O_2 (Merck) for 10 min to block endogenous peroxidase activity to reduce non-specific background staining, followed by a washing step in distilled water.

Antigen-retrieval was performed in a heating Lab Vision PT Module (Thermo Fisher Scientific) using 100 mM citrate buffer, pH = 6.0, for 15 min at 98 °C, followed by a washing step in distilled water and 3 washing steps of 5 min each using TBS containing 0.1% Triton-X (Sigma). Because IHC was not successful to stain all 6 markers in paraffin sections, antigen retrieval conditions were optimized (see below).

Sections were encircled with a PAP pen (Dako) and incubated with TBS containing 3% normal goat serum (Dako) and 0.1% Triton-X for 1 h to further reduce non-specific background staining and for permeabilization of the sections. Sections were subsequently incubated overnight at 4 °C with primary antibodies diluted in Normal Antibody Diluent (Immunologic) as indicated in Table 2. The 6 primary antibodies were tested in a dilution of 1:200 using DAB as chromogen as well as with the optimized dilutions of the primary antibodies as determined in the experiments using cryostat sections.

Secondary HRP antibodies (goat anti-mouse or goat anti-rabbit) that resulted in the best staining of tonsil cryostat sections, were applied as well for IHC staining of paraffin sections (Table 2). Thus, either mono-HRP (Dako) or poly-HRP secondary antibodies (Immunologic) were used, followed by 3 washing steps of 5 min each using TBS. Next, sections were incubated with DAB (Dako) for 10 min, followed by one washing step with tap water to stop the peroxidase enzyme reaction. Sections were then incubated for 30 s in hematoxylin (Sigma-Aldrich) for nuclear counterstaining. Sections were again placed in running tap water for 5 min and then in distilled water. Finally, sections were dehydrated by dipping in 70%, 96% and 100% ethanol (Merck) and 3 times dipping in xylene (VWR Chemicals). Finally, sections were covered with the synthetic mountant Pertex (Histolab).

Control incubations were performed either in the absence of the primary antibody or in the presence of rabbit serum or mouse serum in the same concentration as the primary antibody to determine the effect of the serum on non-specific background staining.

2.5. Variation of antigen retrieval conditions on human paraffin tonsil sections

Two of the 6 proteins (CD31 and CD68) that were successfully stained using IHC on cryostat sections, were not stained using IHC on paraffin sections irrespective the dilution of the primary antibody.

Table 1
Conditions of IHC staining of human tonsil cryostat sections.

Primary antibody	Source	Optimum dilution of primary antibody using AEC	Optimum dilution of primary antibody using DAB	Secondary antibody (mono-HRP-conjugated or poly-HRP-conjugated)
Mouse anti-human CD31	Sanbio[a] (mon6002)	1:200	1:2,000	AEC: poly-HRP DAB: mono-HRP
Mouse anti-human SMA	Dako[b] (1 A4)	1:200	1:2,000	AEC: mono-HRP DAB: mono-HRP
Rabbit anti-human SDF-1α	Abcam[c] (ab9797)	1:200	1:500	AEC: poly-HRP DAB: poly-HRP
Rabbit anti-human CXCR4	Abcam[c] (ab124824)	1:200 (expression hardly visible)	1:200	AEC: poly-HRP DAB: poly-HRP
Mouse anti-human CD68	Dako[b] (M0718)	1:200 (no expression visible)	1:500	AEC: poly-HRP DAB: mono-HRP
Mouse anti-human Ki67	Dako[b] (MIB-1)	1:200 (expression hardly visible)	1:200	AEC: poly-HRP DAB: mono-HRP

Abbreviations: SMA, smooth muscle actin; SDF-1α, stromal –derived factor-1α; CXCR4, C-X-C chemokine receptor type 4; AEC, aminoethyl carbazole; DAB, 3,3′-diaminobenzidine; HRP, horseradish peroxidase.
[a] Sanbio; Uden, The Netherlands.
[b] Dako; Glostrup, Denmark.
[c] Abcam; Cambridge, UK.

Therefore, we determined whether the antigen retrieval procedure (citrate buffer, pH 6.0, for 15 min at 98 °C) was suboptimal. Antigen retrieval conditions were varied as indicated in Table 3. Citrate buffer was compared to Tris-EDTA buffer, pH 9.0. Multiple time points were tested (Table 3). As a positive control, we included endothelial cell staining using a primary antibody against Von Willebrand Factor, which is a routine staining on paraffin sections performed by the Pathology Department at the Academic Medical Center, Amsterdam, The Netherlands.

2.6. Hematoxylin-eosin (HE) staining

For HE staining, human tonsil cryostat sections were air dried for 15 min and paraffin tonsil sections were dewaxed in xylene (VWR Chemicals) and 100% ethanol (Merck). Then, sections were fixed with Formol-Macrodex (4% formaldehyde, 7.2 mM $CaCl_2$, 0.12 M Dextran-70, 0.12 M NaCl and 7.96 mM $CaCO_3$) for 10 min, followed by a washing step in distilled water for 5 min. Nuclei were stained with hematoxylin (Sigma-Aldrich) for 30 s and then, sections were placed in running tap water for 5 min, after which the sections were placed in distilled water. Sections were then stained with eosin (Lot nr.

Table 3
Antigen retrieval conditions tested on human paraffin tonsil sections.

Antibodies	Variations in antigen retrieval conditions	
	Citrate buffer, pH 6.0, 98 °C	Tris-EDTA buffer, pH 9.0, 98 °C
Mouse anti-human CD31	10, 20, 30, 40 min	10, 20, 30, 40 min
Mouse anti-human CD68	10, 20, 30, 40 min	10, 20, 30, 40 min
Rabbit anti-human SDF-1α	10, 20, 30, 40 min	10, 20, 30, 40 min
Mouse anti-human VWF	ND	25 min

Abbreviations: EDTA, ethylenediaminetetraacetic acid; SDF-1α, stromal-derived factor-1α; Tris, tris(hydroxymethyl)aminomethane; VWF, Von Willebrand Factor; ND, not done.

Table 2
Conditions of IHC staining using DAB of human tonsil paraffin sections.

Primary antibody	Source	Optimized conditions obtained from IHC experiments using tonsil cryostat sections	Results of optimized IHC procedure on human tonsil paraffin sections
Mouse anti-human CD31	Sanbio[a]	Primary ab: 1:2,000	No staining even at primary antibody dilution of 1:200
	Cat nr. mon6002	Secondary ab: mono-HRP	
Mouse anti-human SMA	Dako[b] (1 A4)	Primary ab: 1:2,000	Staining
	Ref nr. MO851 Lot nr. 20031819	Secondary ab: mono-HRP	
Rabbit anti-human SDF-1α	Abcam[c]	Primary ab: 1:200	Staining
	Cat nr. ab9797	Secondary ab: poly-HRP	
Rabbit anti-human CXCR4	Abcam[c]	Primary ab: 1:200	Staining
	Cat nr ab124824	Secondary ab: poly-HRP	
Mouse anti-human CD68	Dako[b]	Primary ab: 1:500	No staining even at primary antibody dilution of 1:200
	Cat nr. M0718	Secondary ab: mono-HRP	
Mouse anti-human Ki67	Dako[b]	Primary ab: 1:200	Staining
	Cat nr. MIB-1	Secondary ab: mono-HRP	
Mouse anti-human Von Willebrand Factor	Dako[b]	ND	Staining
	Cat nr. M0616		Primary ab: 1:200 Secondary ab: mono-HRP

Abbreviations: ab, antibody; SMA, smooth muscle actin; SDF-1α, stromal –derived factor-1α; CXCR4, C-X-C chemokine receptor type 4; DAB, 3,3′-diaminobenzidine; HRP, horseradish peroxidase, VWF, Von Willebrand Factor; ND, not done.
[a] Sanbio; Uden, The Netherlands.
[b] Dako; Glostrup, Denmark.
[c] Abcam; Cambridge, UK.

HX264643; Merck) for 20 s, dipped 5 times in distilled water, 15 times in 70% ethanol, 15 times in 96% ethanol and 10 times in 100% ethanol. Afterwards, sections were rinsed 3 times 5 min in xylene. The sections were covered with Pertex (Histolab). All steps were performed at room temp.

2.7. Qualitative and quantitative microscopical analysis of staining patterns

Sections were analyzed qualitatively by light microscopy and images were taken using the Olympus cellSense Standard software and an Olympus BX51 microscope (Leiderdorp, The Netherlands). Staining patterns of the IHC experiments were analyzed by two independent observers (VVVH and CJFVN). Quantitative analysis of cryostat sections and paraffin sections was performed using ImageJ software (Chieco et al., 2013).

3. Results

In the tonsil sections, we focussed on follicles that are packed with white blood cells as is shown in the HE-stained sections (Figs. 1 A, 2 A, 3 A, 4 A).

3.1. IHC on serial cryostat sections: comparison of the chromogens AEC and DAB and mono-HRP-conjugated and poly-HRP-conjugated secondary antibodies

First, the 6 primary antibodies against CD31, SMA, SDF-1α, CXCR4, CD68 and Ki76 were used to compare staining results with AEC and DAB and mono-HRP-conjugated and poly-HRP-conjugated secondary HRP antibodies. The dilution of all primary antibodies was 1:200 (only staining results for CD31 are shown; Fig. 1B-E). AEC as chromogen in combination with mono-HRP secondary antibody resulted in weak staining of endothelial cells (Fig. 1B), whereas the use of poly-HRP-conjugated secondary antibody significantly improved staining of endothelium (Fig. 1C). When using DAB as chromogen, sections were overstained with the anti-CD31 primary antibody in a dilution of 1:200 with both types of secondary antibodies (Fig. 1D, E).

As Fig. 1 shows that DAB is a more sensitive chromogen than AEC, we selected DAB for all further IHC experiments and localized the 6 markers in the follicles of tonsil cryostat sections (Fig. 2). The HE staining shows the morphology of the follicles (Fig. 2A). Since the dilution of 1:200 of the primary antibodies was too low, dilutions of 1:500, 1:1000 and 1:2000 of the primary antibodies were tested (Fig. 2). For CD31 and SMA, a dilution of 1:2000 with the mono-HRP secondary antibody resulted in optimum staining as blood vessels and arterioles/venules were clearly visible in the follicles (Fig. 2B, C).

For SDF-1α and its receptor CXCR4, primary antibody dilutions of 1:500 and 1:200 were optimal, respectively, in combination with poly-HRP-conjugated secondary antibody (Fig. 2D, E). SDF-1α was expressed in and around the follicles and CXCR4 was expressed on almost all white blood cells in follicles (Fig. 2E).

Macrophage marker CD68 and proliferation marker Ki67 were expressed in the follicles with optimal primary antibody dilutions of 1:500 and 1:200, respectively, in combination with mono-HRP-conjugated secondary antibody (Fig. 2F, G). Ki67 was expressed in most white blood cells in the follicles, indicating that white blood cells are rapidly proliferating (Fig. 2G). CD68, on the other hand, was expressed in a subset of white blood cells in the follicles (Fig. 2F).

Table 1 summarizes the optimum incubation conditions for IHC of the 6 proteins in human tonsil cryostat sections. Next, we aimed to utilize those optimized conditions for the 6 primary antibodies (Fig. 2) on serial paraffin sections of human tonsil to determine whether there are differences in staining patterns in cryostat sections and paraffin sections.

3.2. IHC on serial paraffin sections: application of optimized IHC conditions with DAB as chromogen

The IHC conditions optimized on cryostat sections were applied to human tonsil paraffin sections (Fig. 3) and primary antibody dilutions of 1:200 of all 6 primary antibodies in combination with poly-HRP-conjugated secondary antibodies were included as well (Fig. 4). The optimum staining results are summarized in Table 2. For CD31, the primary antibody dilution of 1:2000 in combination with the mono-HRP-conjugated secondary antibody did not result in any positive staining (Fig. 3B) and even a primary antibody dilution of 1:200 in combination with the poly-HRP-conjugated secondary antibody resulted in weak staining only (Fig. 4B). The optimized conditions as determined in cryostat sections for IHC of SMA, SDF-1α, CXCR4 and Ki67 resulted in distinct staining patterns in paraffin sections

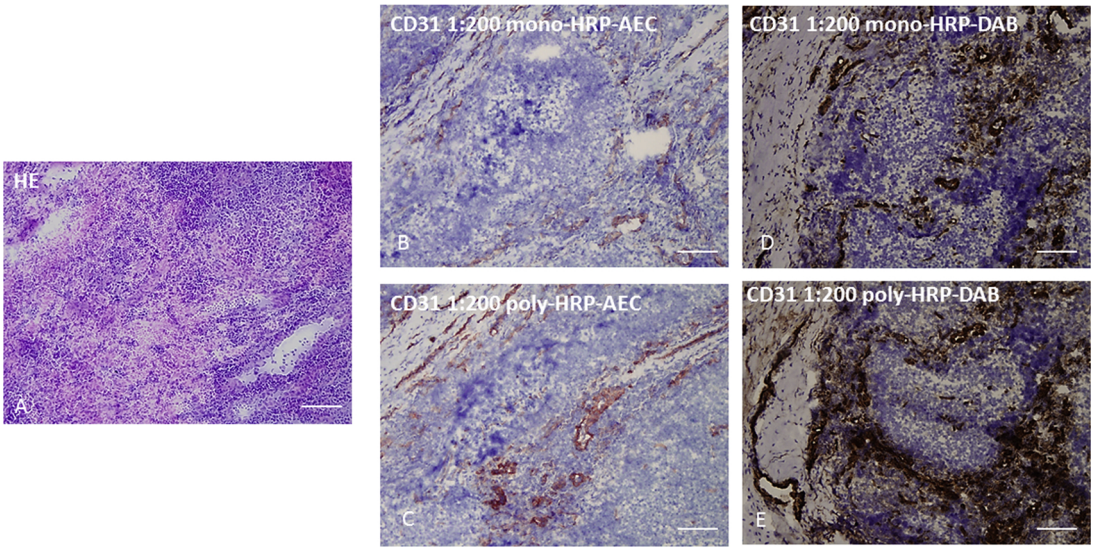

Fig. 1. IHC staining of CD31 in human tonsil serial cryostat sections comparing AEC and DAB as chromogens and mono-HRP-conjugated and poly-HRP-conjugated secondary antibodies. Hematoxylin and eosin (HE) staining shows the follicle area in the tonsil sections that contains white blood cells (**A**). CD31 was expressed in endothelial cells of blood vessels in the follicle. The use of AEC as chromogen resulted only in distinct staining when poly-HRP-conjugated secondary antibody was used (**B, C**). DAB generated stronger staining as chromogen as compared to AEC (**D, E**). Scale bar = 200 μm. Abbreviations: AEC, aminoethyl carbazole; DAB, 3,3′-diaminobenzidine; HRP, horseradish peroxidase.

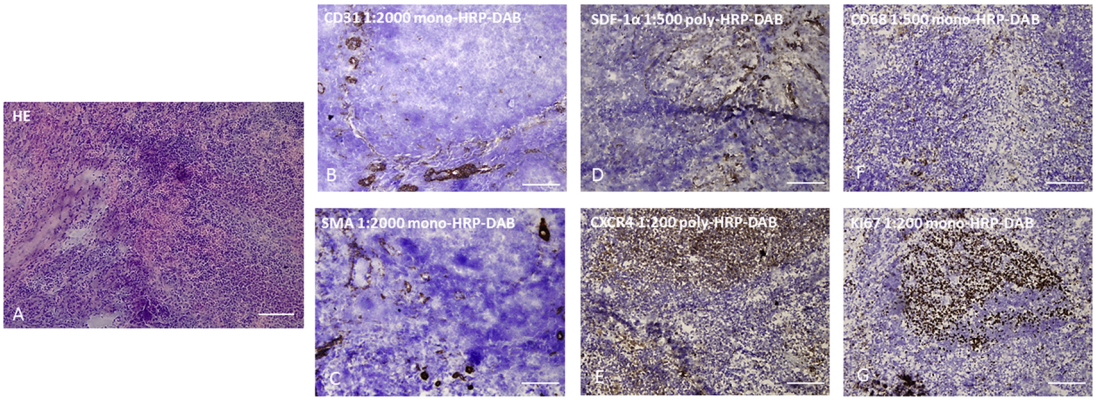

Fig. 2. Optimal IHC staining conditions with the use of DAB as chromogen for staining of human tonsil serial cryostat sections. HE staining shows the morphology of the follicle area in the tonsil sections (**A**). CD31 and SMA were stained optimally using a 1:2000 primary antibody dilution in combination with mono-HRP-conjugated secondary antibody in endothelial cells of blood vessels and smooth muscle cells of arterioles and venules, respectively (**B, C**). Chemoattractant SDF-1α and its receptor CXCR4 were stained optimally in the follicle when using 1:500 and 1:200 primary antibody dilutions, respectively, in combination with poly-HRP-conjugated secondary antibody (**D, E**). CXCR4 was expressed in almost all white blood cells in the follicle (*E*). Macrophage marker CD68 was stained optimally using a 1:500 primary antibody dilution in combination with mono-HRP-conjugated secondary antibody and was expressed in a subset of white blood cells in the follicle (**F**). Proliferation marker Ki67 was stained optimally using a 1:200 primary antibody dilution in combination with mono-HRP-conjugated secondary antibody and was expressed in almost all white blood cells in the follicle (**G**). Scale bar = 200 μm. Abbreviations: SMA, smooth muscle actin; SDF-1α, stromal –derived factor-1α; CXCR4, C-X-C chemokine receptor type 4; DAB, 3,3′-diaminobenzidine; HRP, horseradish peroxidase.

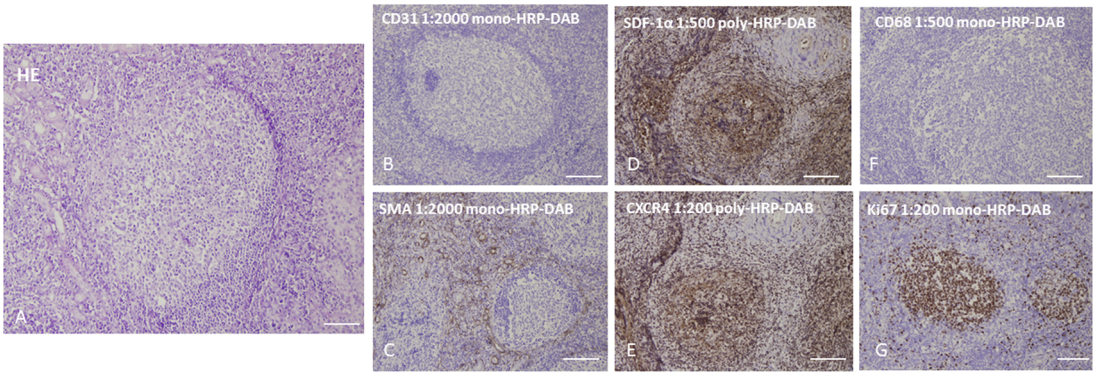

Fig. 3. IHC staining of human tonsil serial paraffin sections using DAB as chromogen and the optimized immunohistochemical conditions obtained from experiments on tonsil cryostat sections. Hematoxylin and eosin (HE) staining shows the follicle area in the tonsil sections that contains white blood cells (**A**). CD31 was not stained using a 1:2000 primary antibody dilution in combination with mono-HRP-conjugated secondary antibody (**B**). SMA was stained in arterioles/venules around the follicle area using a 1:2000 primary antibody dilution in combination with mono-HRP-conjugated secondary (**C**). SDF-1α was abundantly stained in and around the follicle area using a 1:500 primary antibody dilution, in combination with poly-HRP-conjugated secondary antibody (**D**). CXCR4 was stained in almost all white blood cells in the follicles using a primary antibody dilution of 1:200 in combination with poly-HRP-conjugated secondary antibody (**E**). The macrophage marker CD68 did not show positivity in paraffin sections when using a 1:500 primary antibody dilution in combination with mono-HRP-conjugated secondary antibody (**F**). Proliferation marker Ki67 was stained in almost all white blood cells in the follicle using a 1:200 primary antibody dilution in combination with mono-HRP-conjugated secondary antibody (**G**). Scale bar = 200 μm. Abbreviations: SMA, smooth muscle actin; SDF-1α, stromal –derived factor-1α; CXCR4, C-X-C chemokine receptor type 4; DAB, 3,3'-diaminobenzidine; HRP, horseradish peroxidase.

(Fig. 3C–E, G). The images show that the CXCR4-positive and Ki76-positive white blood cells are present in SDF-1α-rich follicles, which are surrounded by SMA-positive arterioles and/or venules (Figs. 3 and 4). CD68 did not show any positive staining with the optimized conditions (Fig. 3F) or even the primary antibody dilution of 1:200 in combination with the poly-HRP-conjugated secondary antibody (Fig. 4F).

3.3. Control incubations

In all tested conditions, control staining in the absence of the primary antibodies was negative in cryostat sections and paraffin sections (Fig. 5).

3.4. Antigen retrieval

In Fig. 6, IHC staining of endothelial cells using anti-CD31 in a cryostat section (Fig. 6A) is compared with that in a paraffin section (Fig. 6B). The same anti-CD31 antibody showed proper staining in cryostat sections but hardly showed any positivity in paraffin sections (Fig. 6A, B). As a positive control, anti-Von willebrand Factor (VWF) was used for IHC staining (provided by the Pathology Department of the Academic Medical Center, Amsterdam, The Netherlands) to detect endothelial cells in serial sections of the same tonsil sample (Fig. 6C). The endothelial cells were clearly positive for VWF (Fig. 6C). The methodological difference between CD31 staining and VWF staining was the primary antibody and the application of citrate buffer and Tris-EDTA buffer for CD31 and VWF staining, respectively.

Therefore, we compared staining of CD31 and CD68 in paraffin sections after antigen retrieval for various periods of time using citrate buffer, pH 6.0 and Tris-EDTA buffer, pH 9.0, respectively (Table 3). SDF-1α was used as a positive control, as it was IHC stained in paraffin sections (Fig. 3D). The data shows that antigen retrieval using Tris-EDTA buffer for 20 min resulted in distinctly stronger staining of SDF-1α compared with antigen retrieval using citrate buffer (Fig. 7). Fig. 8

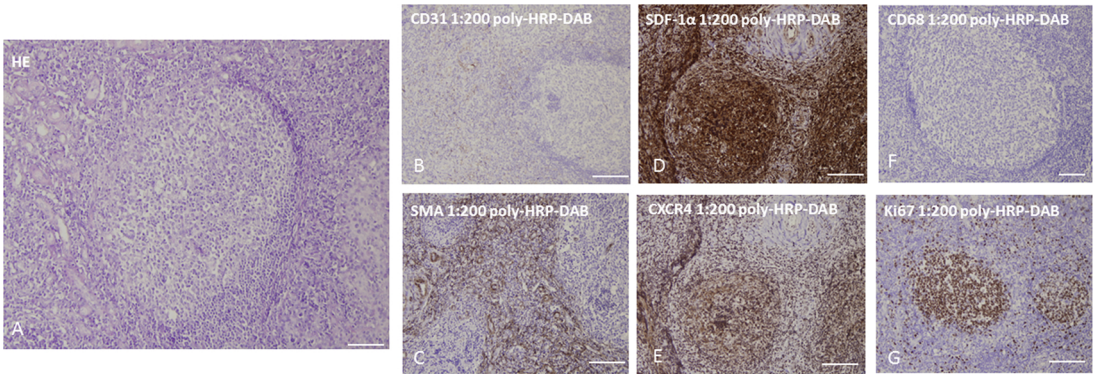

Fig. 4. IHC staining of human tonsil serial paraffin sections using DAB as chromogen and a 1:200 primary antibody dilution for all primary antibodies in combination with poly-HRP-conjugated secondary antibody. Hematoxylin and eosin (HE) staining shows the follicle area in the tonsil sections that contains white blood cells (**A**). CD31 showed weak staining in some endothelial cells of blood vessels around the follicle (**B**). SMA was expressed in arterioles/venules in and around the follicle (**C**). SDF-1α and CXCR4 were abundantly stained in and around the follicle area and CXCR4 was expressed on almost all white blood cells in the follicle (**D, E**). The 1:200 primary antibody dilution of anti-SDF-1α was too low, as the staining was too strong (**D**). The macrophage marker CD68 did not show positivity in paraffin sections (**F**). Ki67 was expressed in the white blood cells in the follicle (**G**). Scale bar = 200 μm. Abbreviations: SMA, smooth muscle actin; SDF-1α, stromal –derived factor-1α; CXCR4, C-X-C chemokine receptor type 4; DAB, 3,3'-diaminobenzidine; HRP, horseradish peroxidase.

shows the comparison of both buffers for antigen retrieval during 20 min on the one hand and the staining of cryostat sections without antigen retrieval on the other hand for CD31 (Fig. 8A-C), CD68 (Fig. 8D-F) and SDF-1α (Fig. 8G-I). The IHC staining results of paraffin sections show that CD31 staining remained weak irrespective the buffer and the period of time used for antigen retrieval (Fig. 8A, B) whereas CD68 was not stained at all (Fig. 8D, E), whereas on cryostat sections, a distinct staining of CD31 and CD68 was obtained using the same antibodies (Fig. 8C, F).

3.5. Tissue morphology in cryostat sections and paraffin sections

High magnifications of Ki67 staining in follicles in a cryostat section (Fig. 9A) and a paraffin section (Fig. 9B) show the morphological superiority of tissues in paraffin sections. Moreover, we have analysed quantitatively the size of the nuclei of cells in follicles to establish any difference in size due to tissue swelling or shrinkage in the procedures to obtain cryostat sections or paraffin sections. We did not find any difference between diameters of nuclei in the Ki67-stained cells in cryostat sections or paraffin sections, indicating that tissue swelling or tissue shrinkage did not occur,

4. Discussion

In this study, we critically compared IHC staining of specific proteins in follicles of human tonsils in serial cryostat sections and serial paraffin sections with the use of AEC and DAB as chromogens, as well as mono-HRP-conjugated and poly-HRP-conjugated secondary antibodies to obtain optimum staining. We defined optimum staining as the situation when there is a high contrast between the positive tissue

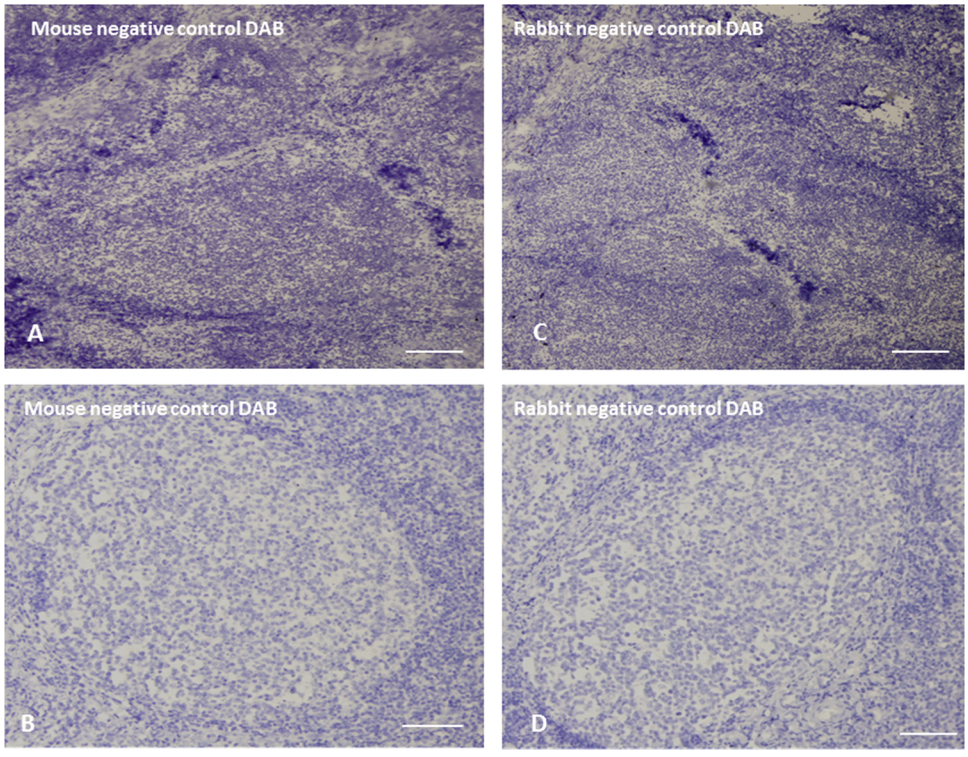

Fig. 5. Negative control incubations in human tonsil serial cryostat sections (**A, C**) and human tonsil paraffin serial sections (**B, D**) in the absence of primary antibodies and in the presence of goat anti-mouse (**A, B**) or goat anti-rabbit (**C, D**) poly-HRP-conjugated secondary antibodies for DAB as chromogen. Scale bar = 200 μm. Abbreviations: AEC, aminoethyl carbazole; DAB, 3,3'-diaminobenzidine; HRP, horseradish peroxidase.

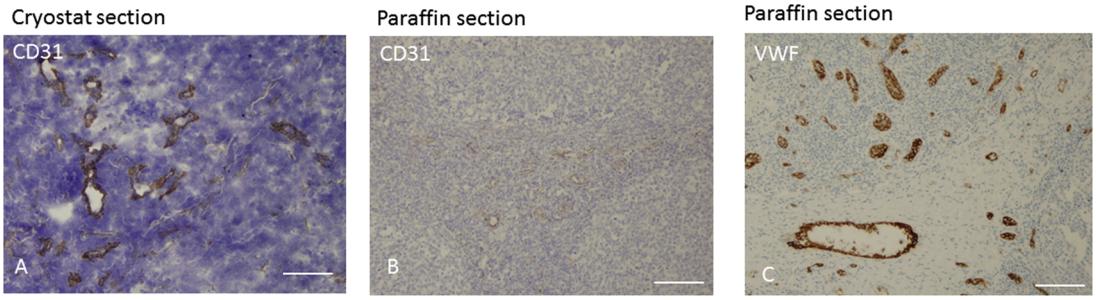

Fig. 6. Comparison of CD31 staining in human tonsil cryostat sections versus human tonsil paraffin sections using DAB as chromogen. In human tonsil cryostat sections, CD31 was expressed in endothelial cells of blood vessels around the follicles using a primary antibody dilution of 1:2000 in combination with mono-HRP secondary antibody (**A**). In human tonsil paraffin sections, CD31 expression was hardly visible (**B**). VWF staining on paraffin sections was used as a positive control (**C**). Scale bar = 200 µm. Abbreviations: DAB, 3,3'-diaminobenzidine; HRP, horseradish peroxidase; VWF, Von Willebrand Factor.

structures (test) and background (control) in the presence and absence of primary antibodies. This is exemplified by the difference between the staining after control incubations as in Fig. 5B, D versus test incubations as in Fig. 3B-G. Fig. 10 illustrates in a flowchart how optimum staining can be obtained.

We tested 6 primary antibodies against proteins that are known to be highly expressed in human tonsil, endothelial cell marker CD31 (Jackson et al., 2000), smooth muscle cell marker smooth muscle actin (SMA) for arterioles and venues (Honma et al., 1995), chemokine stromal-derived factor-1α (SDF-1α) and its receptor C-X-C receptor type 4 (CXCR4) (Corcione et al., 2000; Ehlin-Henriksson et al., 2006), macrophage marker CD68 (Jayakumar et al., 2005) and proliferation marker Ki67 (Hsu et al., 2013).

CD31 and SMA were found to be localized around the follicles in the tonsil sections (Fig. 2B, C). SDF-1α is an important chemoattractant for homing of CXCR4-positive white blood cells in the follicles (Corcione et al., 2000), explaining the high abundancy of SDF-1α and CXCR4 expression in follicles (Figs. 2D, E, 3 D, E, 4 D, E). Proliferation marker Ki67 was expressed in nuclei of almost all white blood cells within follicles (Figs. 2G, 3 G, 4 G), indicating that these cells are rapidly proliferating cells as was previously reported by (Hsu et al., 2013). Nuclear staining was more evident in paraffin sections (Figs. 3G, 4 G) than in cryostat sections (Fig. 2G), in particular because structures in cells outside nuclei were also Ki67-positive in cryostat sections (Fig. 9).

IHC on serial cryostat sections showed that DAB is clearly a more sensitive chromogen than AEC for peroxidase-dependent IHC production of water-insoluble coloured reaction product (Fig. 1). Table 1 shows the enormous difference in sensitivity of IHC staining when AEC or DAB are used. These findings are totally in agreement with the systematic investigations of Trojanowski et al. (1983) and De Jong et al. (1985) who both concluded three decades ago that HRP in combination with DAB was the most sensitive chromogenic visualization system for IHC and it still is. Another advantage of DAB is that the staining is permanent, whereas staining with AEC fades in time (Shen and Wu, 2015).

The primary antibodies against CD31 and CD68 showed protein expression in cryostat sections (Fig. 2B, F), but not in paraffin sections (Figs. 3B, F, 4 B, F). The notion that some proteins were clearly positively stained in cryostat sections but not in paraffin sections, can be explained by loss of antigenicity due to formaldehyde fixation and/or the high temperature (60 °C) during paraffin embedding (Chen et al., 2010; Kumar et al., 2015; Shi et al., 1997, 2011). Besides, epitopes are masked by protein crosslinks after fixation, which impedes antibody binding. Therefore, antigen retrieval is a crucial step when dealing with paraffin sections to dissolve protein crosslinks, which enables the antibodies to bind to their epitopes (Chen et al., 2010; Kumar et al., 2015; Ramos-Vara, 2005; Shi et al., 2011). Antigen retrieval is often performed using citrate buffer, pH 6.0, at 98 °C for 15–20 minutes (Chen et al., 2010; Kumar et al., 2015). In the case of CD31 and CD68, other antigen retrieval methodology was probably required, as has been suggested (Ghatak and Combs, 2014; Shi et al., 1997, 2011). However, we tested antigen retrieval incubation times of 10–40 min and replacement of citrate buffer, pH 6.0 by Tris-EDTA buffer, pH 9.0 for 10–40 min but IHC staining of CD31 and CD68 was not improved (Fig. 8). This implies that antigen retrieval is not always the ultimate solution for unsuccessful IHC staining when using paraffin sections (Figs. 6 and 8). Fig. 6 shows that IHC staining of CD31 for the localization of endothelial cells was successful in cryostat sections (Fig. 6A), but not in paraffin sections after antigen retrieval (Fig. 6B). However, when anti-VWF was used as primary antibody for endothelial cell detection, endothelial cells of all blood vessels were clearly positive in paraffin sections (Fig. 6C). This shows that antigen retrieval cannot always provide access of the primary antibody to epitopes, explaining why application of anti-CD31 did not result in a proper staining whereas that of anti-VWF did (Fig. 6).

Since antigen retrieval is not required for IHC on cryostat sections,

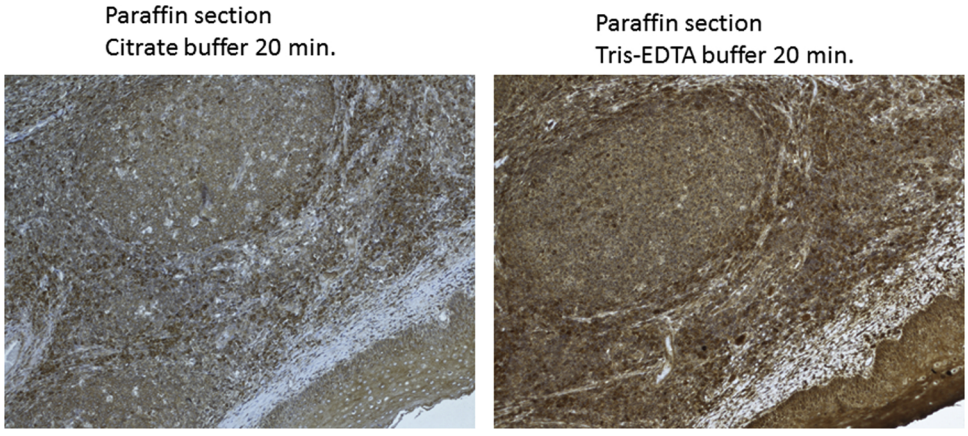

Fig. 7. Comparison of SDF-1α staining in human tonsil paraffin sections using citrate buffer versus Tris-EDTA buffer for antigen retrieval with DAB as chromogen. SDF-1a staining was weak in tonsil paraffin sections when citrate buffer, pH 6.0, was used for antigen retrieval for 20 min (**A**), whereas the staining was optimal when Tris-EDTA buffer, pH 9.0, was used for antigen retrieval for 20 min (**B**). Scale bar = 200 µm. Abbreviations: DAB, 3,3'-diaminobenzidine; EDTA, ethylenediaminetetraacetic acid; SDF-1α, stromal-derived factor-1α; Tris, tris(hydroxymethyl)aminomethane.

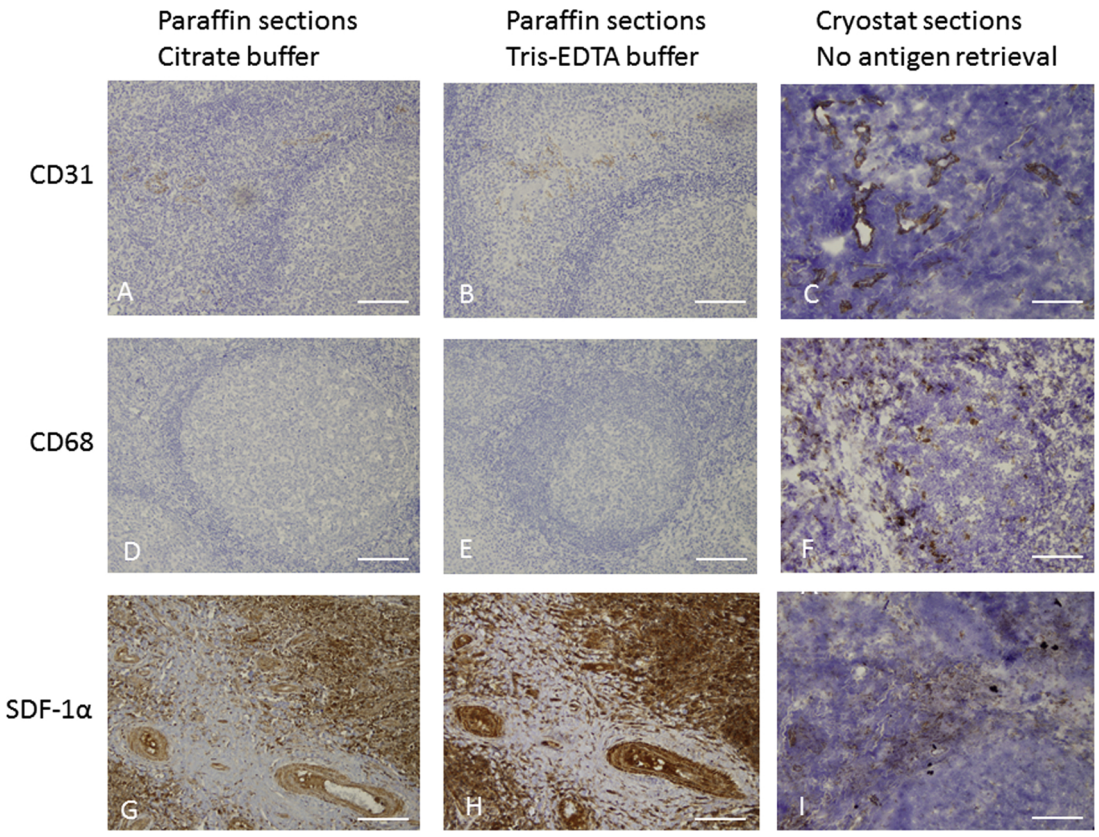

Fig. 8. Comparison of CD31, CD68 and SDF-1α staining with DAB as chromogen on tonsil paraffin sections using antigen retrieval versus tonsil cryostat sections without antigen retrieval. CD31 shows weak staining in paraffin tonsil sections (**A, B**) and strong staining in tonsil cryostat sections (**C**). CD68 did not show any positivity in tonsil paraffin sections (**D, E**), whereas a subset of white blood cells was positive for CD68 in tonsil cryostat sections (**F**). SDF-1α staining was performed as a positive control and was expressed in both paraffin sections and cryostat sections (**G-I**). Scale bar = 200 µm. Abbreviations: DAB, 3,3'-diaminobenzidine; SDF-1α, stromal –derived factor-1α.

there is a higher chance for successful staining. However, the tissue morphology in cryostat sections is inferior to that in paraffin sections (compare Figs. 1A and 2 A with 3 A and 4 A). On the one hand, tissue morphology is far better preserved in paraffin sections (Chen et al., 2010; Kumar et al., 2015) as is shown in Fig. 9, but for IHC cryostat sections have the advantage that there is no loss of antigenicity due to formaldehyde fixation or heat during paraffin embedding (Chen et al., 2010; Kumar et al., 2015; Shi et al., 1997, 2011).

Cryostat sections were 7-µm thick while the paraffin sections were 5-µm thick. Thinner sections usually have superior morphology and less background staining. However, in practice, intact 5 µm-thick cryostat sections are hard to generate particularly when serial sections are required. The difficulty of sectioning 5 µm-thick cryostat sections depends also on the type of tissue. For example, sectioning of liver tissue is relatively easy, compared to tissues rich in connective tissue, such as tonsil. Therefore, we consider 7 µm thickness of tonsil sections as a minimum. On the other hand, 2–5 µm thickness is the optimum for paraffin sections and also conventionally applied. So, the difference in section thickness is inherent to the application of cryostat sections versus paraffin sections. We therefore used cryostat sections that were as thin as possible (7 µm) and paraffin sections that were as thick as is conventional (5 µm) to optimize the comparability between the two methods.

In conclusion, our IHC study using cryostat and paraffin human tonsil sections shows that DAB is the more sensitive chromogen as compared to AEC for peroxidase-dependent chromogenic IHC staining.

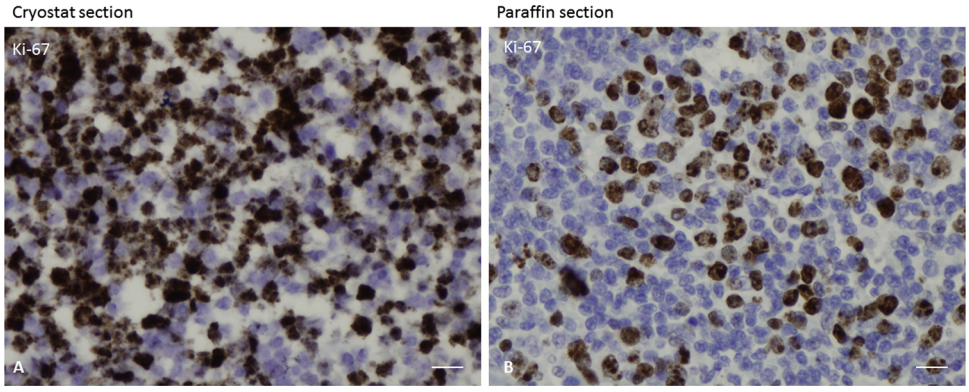

Fig. 9. Morphological comparison of a cryostat section (**A**) and a paraffin section (**B**) after Ki67 staining using DAB as chromogen. Scale bar = 50 µm.

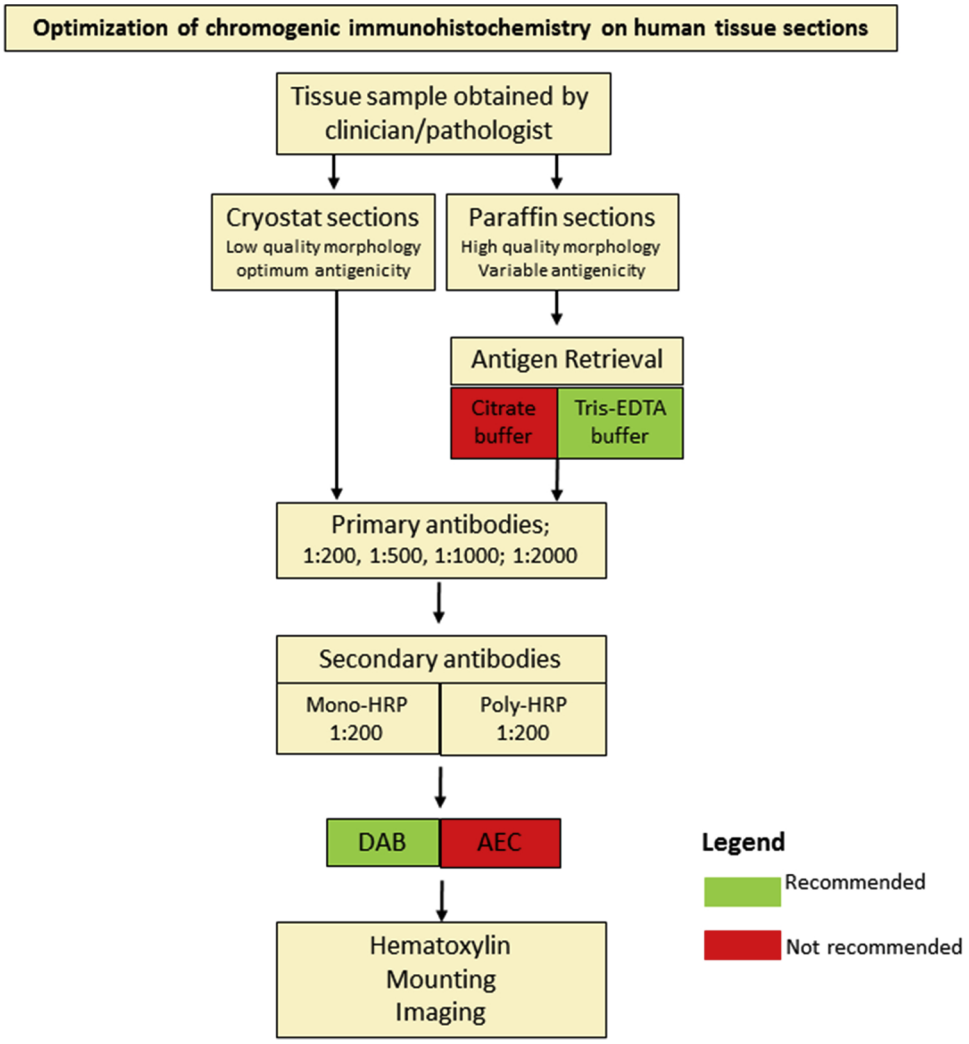

Fig. 10. Flowchart of the optimization procedures for chromogenic immunohistochemistry on human tissue sections.

In addition, IHC on cryostat sections results in successful staining whereas in paraffin sections loss of antigenicity and masking of epitopes by crosslinking can impede IHC staining. Clearly, antigen retrieval does not guarantee successful IHC staining. Therefore, testing of multiple primary antibodies is recommended when IHC staining of a novel protein is being developed. The application of mono-HRP-conjugated or poly-HRP-conjugated secondary antibodies has to be selected depending on the affinity of the primary antibody for its antigen. Finally, tissue morphology in paraffin sections is superior to that in cryostat sections, indicating that the choice for unfixed frozen tissue versus formalin-fixed paraffin-embedded tissue has to be made on the basis of trial-and-error.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

VVVH: Designed the study, performed experiments, analysed and interpreted the data, gave intellectual input, revised the manuscript, approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

ALJ, KF and MK: Performed experiments, analysed data, approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

RJM: interpreted the data, gave intellectual input, revised the manuscript, approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

CJFVN: Designed the study, supervised the entire study, analysed and interpreted the data, gave intellectual input, revised the manuscript, approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

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