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DOI: https://doi.org/10.1016/j.acthis.2023.152127

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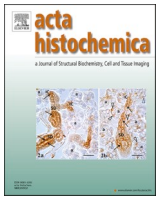
Acta Histochemica 126 (2024) 152127

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.com/locate/acthis



Delineation of the healthy rabbit tongue by immunohistochemistry – A technical note

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ARTICLE INFO

Keywords:
Cryosection
Antigen retrieval
Immunofluorescence
Salivary glands
Excretory ducts

ABSTRACT

In the oral cavity the tongue is an important muscular organ that supports the swallowing of food and liquids. It is responsible for the sense of taste, based on the many different taste buds it contains. Research in the field of tongue diseases demands for suitable preclinical models. The healthy rabbit tongue may therefore serve as baseline and reference for the pathological situation. With this consideration, we covered the fixation and histological stainings as well as the immunohistochemical labelling of the healthy rabbit tongue. In this technical note, initial choice of the fixative is discussed, with a comparison of formalin fixation and subsequent paraffin embedding versus cryopreservation. Moreover, we delineate the effect of an antigen retrieval step for formalin fixation by several examples. Finally, we provide ECM markers collagen I, collagen III, fibronectin, α-SMA and elastin staining as well as ki67 for proliferative status and PAR-2 protein expression as a marker for inflammatory status and nociception in tongue sections, mainly from the tongue body. Technically, we found superiority of paraffin sections for collagen I, collagen III, fibronectin, ki67 and α-SMA labelling, for selected detections systems. As for ECM components, the lamina propria was very rich in collagen and fibronectin, while the muscular body of the tongue showed only collagen and fibronectin positive areas between the muscle fibers. Moreover, α-SMA was clearly expressed in the walls of arteries and veins. The inflammatory marker PAR-2 on the other hand was prominently expressed in the salivary glands and to some extent in the walls of the vessels. Particular PAR-2 expression was found in the excretory ducts of the tongue. This technical note has the aim to provide baseline images that can be used to compare the pathological state of the diseased rabbit tongue as well as for inter-species comparison, such as mouse or rat tongue. Finally, it can be used for the comparison with the human situation.

1. Introduction

The tongue is an important part of the oral cavity and enables swallowing of liquids and food by diverse muscle activity (Lobprise and Wiggs, 1993). As a chiefly muscular organ with a rich vascular supply (Hellekant, 1976), the tongue is capable of stretching and shortening, but also of tipping up or curling down. During chewing, the tongue keeps the food bolus between the teeth and subsequently moves it to the pharyngeal region before it is swallowed. With its taste buds, the tongue is responsible for the sense of taste (sweet, bitter, sour, salty and umami (Fan et al., 2022) – and less studied taste qualities like chemesthesis (Roper and Chaudhari, 2017)). The taste buds include columnar sensory cells embedded in the stratified epithelium of the tongue (Roper and Chaudhari, 2017). Also, the tongue enables phonation and vocalization – and the word *tongue* is a synonym for *language*. Furthermore, in rodent animals like rabbits, the tongue serves for grooming, parasite control and wound cleaning. In addition, rabbit mothers nurse their neonatal pups by licking their fur and thus stimulating the activation of dopaminergic neurons (Aguirre et al., 2017).

It is important to know the normal anatomy of the healthy rabbit tongue in order to be able to deduce abnormalities and diseases in this preclinical animal model. There are different kinds of tongue diseases, such as the fissured tongue, acute pseudomembranous *candidiasis*, and different kinds of *glossitis* (Joseph and Savage, 2000). In research, the rabbit tongue has served for developmental studies in order to determine the period of formation of the lingual and circumvallate papillae,

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https://doi.org/10.1016/j.acthis.2023.152127
Received 11 May 2023; Received in revised form 23 November 2023; Accepted 23 November 2023
Available online 30 November 2023

the thickening of lingual epithelium and the occurrence of the primordia of foliate papillae as well as the fungiform and filiform papillae (Haddad et al., 2021).

The study of cancers that commonly requires surgical resection of parts of the tongue may also be realized by the rabbit tongue model, where muscle regeneration is a key topic in regenerative healing (MacDonald et al., 2022). Furthermore, tongue flaps are commonly used to address the reconstruction of many oral, pharyngeal, and perioral defects (Strauss and Kain, 2014). In addition, modeling of lingual function and dysfunction is of paramount importance for the understanding of the nervous system that controls tongue function for this complex behavior (Rudisch et al., 2022). Finally, also tongue coating (halitosis) studies may be realized with the rabbit tongue model, where the microbial composition of the coating as well as its impacts on the general health can be assessed (Seerangaiyan et al., 2018).

For such experimental approaches, we provide a basis of different histological stainings and immunohistochemical labellings of the healthy rabbit tongue tissue. First, we compare the fixation techniques of either paraffin embedding or cryoembedding for collagen I, collagen III, fibronectin, α-SMA and ki67; then we address the antigen retrieval step for the paraffin embedded sections to improve antigen retrieval; and finally we provide different serial sections in order to show several stainings of the same target structure in detail, such as the salivary glands or excretory ducts. A special immunohistochemical staining presented here is the protease-activated receptor-2 (PAR-2) immunohistochemical labelling. PAR- 2 is an inflammatory marker, one of four PARs (PAR-1 to PAR-4), that is abundantly recognized for its role in the development of chronic inflammatory diseases (Heuberger and Schuepbach, 2019). PAR-2 expression in the healthy rabbit tongue may be important in the associated inflammatory status of tongue diseases (for example *glossitis*) and potential crosstalk between taste and immune signalling pathways (Wu, 2021).

As such, our technical note provides many different images of the healthy rabbit tongue, mainly from the tongue body, that can be compared to the diseased status of the rabbit tongue model as well as to other rodent models, primates (Iwasaki et al., 2019) or the human situation (Emmanouil-Nikoloussi and Kerameos-Foroglou (1992); Moayedi et al., 2021). Covering all different diseased states of the tongue would be beyond the scope of this technical note, so the focus lies on the healthy rabbit tongue.

2. Materials and methods

2.1. Tongue extraction

Rabbit tongue was received from a dead female New Zealand White rabbit that was included in a calvarial bone defect project. This corresponding project was licenced by the Animal Ethics Committee at local authorities (Canton Zurich ZH 115/2015;090/2021 and ZH 080/21) (Ghayor and Weber (2018); Siegenthaler et al., 2020). The whole tongue was used, as received after isolation from this cadaver. Then, following storage on ice for 20 min and sectioning (mainly the tongue body was used; in addition, for a special question lingual prominence large posterior area was also considered), it was ready for histology. The tongue was sectioned in frontal planes.

2.2. Histology and Immunohistochemistry

The tongue pieces were cut in 2 parts for either paraffin embedding or cryopreservation. Paraffin embedding included fixation in formalin for one day, dehydration, paraffin-embedding and sectioning into 5-μm-thick slices. Before they were stained, paraffin embedded sections were deparaffinized utilizing xylene and rehydrated (descreasing gradient of ethanol). As for the cryoembedded samples, they were embedded in Tissue-Tek® O.C.T. (Sakura, Alphen aan den Rijn, The Netherlands, Europe). Then, they were frozen before cryosections of 5 μm thickness were cut with a microtome. Subsequently, they were thawed and fixed with formalin for 10 min, and finally washed with 1xTBS, followed by IHC procedures.

Table 1
Antibodies and conditions for IHC labellings.

Primary antibody	*Supplier*	*Dilution*
Mouse monoclonal anti-collagen I	ab90395; Abcam, Lucerne, Switzerland	1:200
mouse monoclonal anti-collagen III	AF5810; Acris, Wettingen, Switzerland	1:200
Mouse monoclonal anti-α-SMA	A2547; Sigma-Aldrich, Buchs, Switzerland	1:500
mouse monoclonal anti-fibronectin	F0791; Sigma-Aldrich, Buchs, Switzerland	1:200
mouse monoclonal anti-ki67	NBP2–22112; Novus Biologicals	1:500
mouse monoclonal anti-PAR-2	Santa Cruz Biotechnology, sc-13504 (SAM11) on Refine-kit (anti-Rabbit-Polymer) and histofine-Mouse Polymer	1:250 1:50
Normal Mouse Serum; Isotype Negative Control	08–6599, Invitrogen	no dilution

According to general protocols, Elastica van Gieson (EL), Masson Goldner Trichrome (GT) and Haematoxylin&Eosin (HE) stainings were performed. For IHC, an antigen retrieval (AR) step was performed for paraffin sections, using 10 mM citrate buffer (pH 6.0) with 0.05% Tween-20 for 20 min at 95 °C. In some cases, no AR was made in order to generate a control, for the comparison of conditions with and without AR. For certain epitopes, such as ki67 and α-SMA, corresponding sections were permeabilized with 0.5% Triton X-100 in 1xTBS for 10 min, followed by three times washing with 1xTBS. After that, the sections were blocked in 5% donkey serum and 1% BSA in 1xTBS for 1 h (at room

Table 2
Overview of markers, techniques and stainings used in this study. Key: ECM = Extracellular Matrix, Chro = Chromogenic detection, Fluo = Immunofluorescence, AR = antigen retrieval. Note that for Fibronectin and mouse isotype negative control (NC) images with and without AR-step are shown; "AR" and "no AR" is mentioned on these images and AR was only performed in paraffin sections. For all other stainings on paraffin sections there is no mention about AR, but AR was performed.

Marker	*Description/ Purpose*	*Fixation*	*AR*	*Detection System*
Collagen I (Col I)	Structural protein in ECM	Para + cryo	Yes	Chro + Fluo
Collagen III (Col III)	Structural protein in ECM	Para + cryo	Yes	Chro + Fluo
Fibronectin (Fn)	Glycoprotein for network building of the ECM	Para + cryo	Yes + No	Chro + Fluo
alpha smooth muscle actin (α-SMA)	Contractile smooth muscle fibres in ECM	Para + cryo	Yes	Fluo (Chro)
ki67	Cell nucleus protein expressed in proliferating cells	Para + cryo	Yes	Chro (Fluo)
PAR-2	Inflammation-related protein on cell surface	Para	Yes	Chro
Elastica van Gieson (EL)	Elastic fibers	Para	(classical histology)	(classical histology)
Masson Goldner Trichrome (GT)	Connective tissue structures	Para	(classical histology)	(classical histology)
Haematoxylin&Eosin (HE)	Most structures for comprehensive structural overview	Para	(classical histology)	(classical histology)
Isotype Negative Control (NC)	Normal Mouse Serum	Para	Yes + No	Chro + Fluo

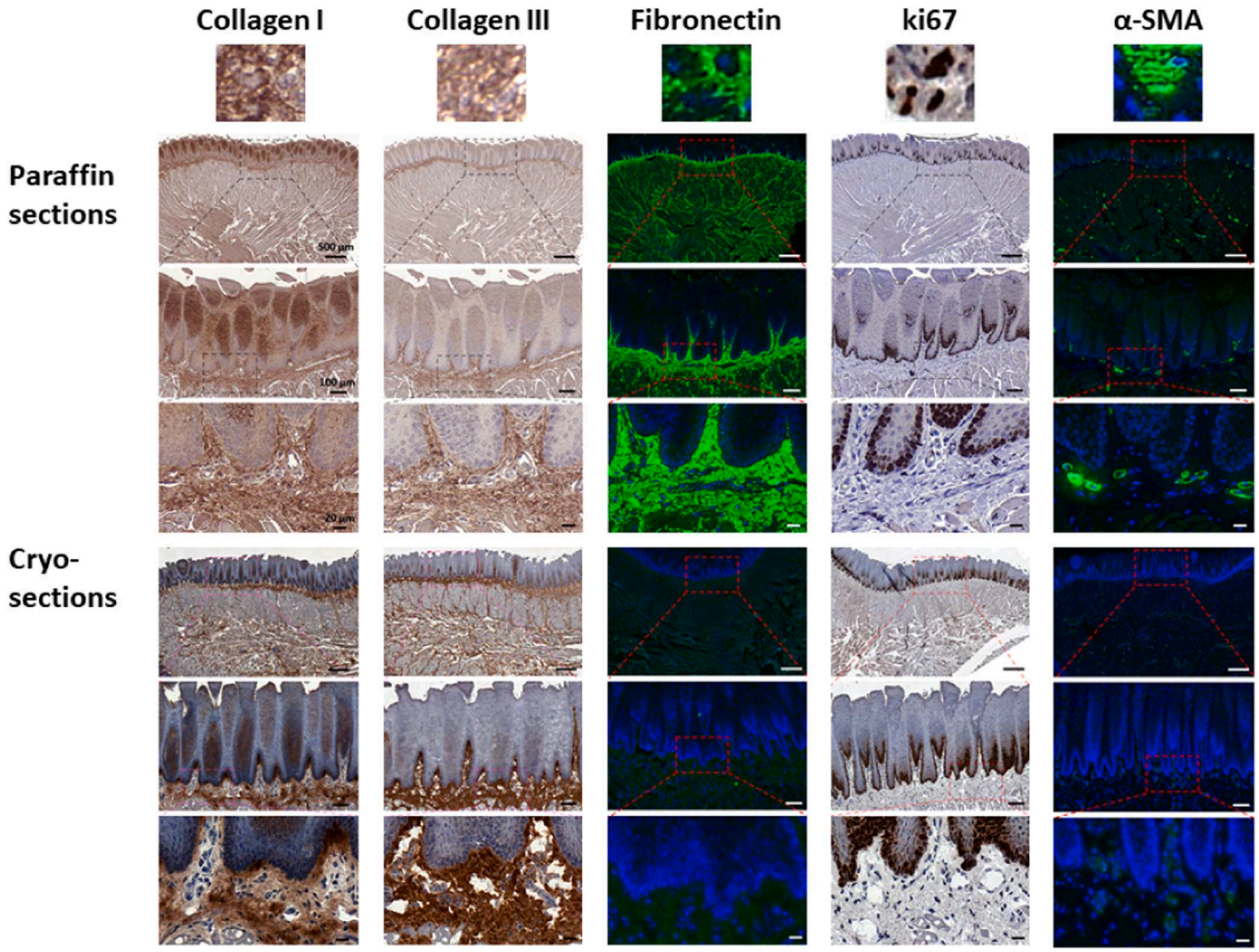

Fig. 1. Collagen I, collagen III, fibronectin, ki67 and α-SMA immunoshistochemical labelling with DAB as chromogen (dark brown colour) or with immunofluorescence detection (light green) and cell nuclei (DAPI labelling, blue) for paraffin sections with antigen retrieval (AR, upper row) and for cryosections (lower row) of the tongue body. Corresponding inserts (dashed line) depict area used for images at the next lower level.

temperature (RT)). Next, sections were incubated with different antibodies, as reported previously (Meier Bürgisser et al., 2023) (Table 1).

Dilutions of antibodies were either taken from technical sheet data, provided by the suppliers; or by titration of different concentrations and comparison of staining intensities. The laboratory validation of PAR-2 antibody had been performed before (Meier Bürgisser et al., 2020). Normal Mouse Serum Control (08–6599, Invitrogen, no dilution) was used; the corresponding sections were incubated with it (4 °C overnight) and used as a negative control for all antibodies.

Fluorescent IHC was performed for collagen I and III, fibronectin and α-SMA, respectively (ki67 for control). Chromogenic IHC labelling was conducted for collagen I and III, fibronectin, ki67 and PAR-2, respectively (α-SMA for control) as reported previously (Meier Bürgisser et al., 2021; Meier Bürgisser et al., 2022; Meier Bürgisser et al., 2023).

For chromogenic IHC, samples were blocked with 3% hydrogen peroxide solution in water for 10 min (RT) and subsequently washed 3 times with 1xTBS. Primary antibody detection was made with a biotinylated anti-mouse IgG secondary antibody and streptavidin-horseradish peroxidase (HRP) (ZytoChem Plus HRP Kit Mouse; Zytomed Systems, Muttenz, Switzerland). Then, colorimetric detection was conducted according to the manufacturer's protocol with DAB (DAB Substrate Kit High Contrast; Zytomed Systems, Germany). Last, slides were given a wash in tap water and mounted, using Faramount Aqueous Mounting Medium from Agilent.

With a slide scanner (Pannoramic 250 Flash II, 3Dhistech, Budapest, Hungary), images of whole tissue sections were taken. We made snapshots of fields of view (FOVs) with CaseViewer-Sofware v.2.1 or imaged them with a Leica 6000 light microscope (Leica, Basel, Switzerland). The techniques and stainings used in this study are summarized in Table 2.

3. Results and discussion

3.1. *What has to be favoured – formalin fixation or cryopreservation?*

Finalizing animal experiments, researchers in preclinical surgery need to decide which kinds of histology and immunohistochemical labelling they want to utilize in order to get optimum readouts from their histological sections. Starting with the freshly harvested tissue, the first decision relies on the fixation technique, and formalin fixation with subsequent paraffin embedding has to be compared with cryopreservation. HE staining can be used well after both of these techniques as a usual, easy and low-cost staining. In contrast, if immunohistochemical labelling is decided for, there may be differences in the outcomes. Images may not only differ in colour intensity, but also in the precision of distinct structures, with often blurred boundaries occurring, which impedes proper delineation. It has to be noted that any fixation method

inactivates enzymes to some extent, along with more or less prominent changes in the secondary and tertiary enzyme structures (Fowler et al., 2008), however, full denaturation should be prevented (Tsutsumi, 2021).

Furthermore, with regard to achieve tissue stabilization by the crosslink of extracellular matrix (ECM) components that is necessary for the thin tissue sections (3–10 μm), the fixative under view can have an important impact. Depending on what has been chosen, molecular bridging in the fixed tissue can be different, along with partial breakdown of the cell membranes. As such, the permeabilisation of the cells and thereby the access for specific antibodies into these cells may be completely different depending on the fixative of choice – and may be seminal for a decent immunohistochemical outcome.

Next to formalin fixation and cryopreservation other fixation methods have been reported, such as paraformaldehyde-lysine-periodate, the Bouin solution, or acetic formalin (Salguero et al., 2001). Furthermore, there are antigen retrieval (AR) steps in order to retrieve the loss of antigenicity which will theoretically return proteins to their prefixation conformation. In order to reach antigens inside cells, permeabilisation chemicals are at hand, such as the detergents TritonX-100, Tween20, glycine and hydrogen peroxide (Rosas-Arellano et al., 2016). Moreover, protocols for enzymatic AR and heat-induced epitope retrieval in a microwave oven, a pressure cooker or a steamer have been reported (Ramos-Vara, 2017).

Here, we compared formalin fixation with a 4% paraformaldehyde solution to cryopreservation from a healthy rabbit tongue tissue. We hypothesized that cryopreservation would be superior to formalin fixation. However, as will be shown, the outcome depended on the immunohistochemical target structure and on the detection system, where we compared chromogenic and fluorescent detection, respectively.

In Fig. 1, a rabbit tongue body section was stained for collagen I, collagen III, fibronectin, ki67 and α-SMA, respectively, and is presented in different magnifications. In comparison with amino ethyl carbazole (AEC), the chromogenic detection system based on DAB has been reported to be superior (Hira et al., 2019). Obvious differences can be seen in the collagen I stained sections; while in the paraffin sections besides the positively dark brown structures there is a lot of brown background staining in the rest of the tissue, much more distinctive and definable structures can be delineated in the cryosection, because of the rather bluish colour of the collagen I negative tissue areas.

In the cryosections, particularly the epithelium and the lingual filiform papillae with the taste buds are stained bluish and grey in contrast to the paraffin embedded sections, where they appear in predominantly brown colour. Moreover, the cells in the lamina propria are stained dark blue in the cryosections, whereas they are light blue to greyish in the paraffin sections – again leading to less contrast with respect to the surrounding structures and hence less distinctness.

Despite these differences, it can be clearly seen in both types of fixation methods that collagen I positive areas are found in parts of the filiform papillae and in the ECM of the lamina propria. Moreover, the muscle body with the vertical and transverse muscle fibers exhibits partial brown staining, detectable as fine lines around the muscle fibrils in the provided frontal section, which are associated with the endomysium.

As for the collagen III staining, the collagen III positive areas are confined to the lamina propria and the endomysium, however not to the lingual filiform papillae as found for collagen I. In contrast to collagen I stained sections, collagen III distribution is better visible in the paraffin sections, at least at higher magnifications (Fig. 1), where the distinct structures that are positive for collagen III are better visualized. In the collagen III stained cryosections, there is a very dark brown and strong staining, so that at higher magnifications it turns out to be a disadvantage. However, collagen III staining for overviews might nevertheless be better in cryosections (low magnification, lower row: upper line Fig. 1).

The frontal plane tongue images immunohistochemically labelled for fibronectin exhibit particularly impressive fluorescent labelled structures in the paraffin sections, while the cryosections do not provide such a high green-to-black contrast (Fig. 1). The fibronectin distribution between the muscle fibrils can be attributed to the connective tissue and the endo- and paramysium of the vertical and horizontal muscle fibers. In addition, the lamina propria of mucosal folds with diffuse lymphatic tissue is rich in fibronectin, which can be determined very well in the higher magnified images of the paraffin sections, but only vaguely in the corresponding cryosections. Particularly, the lamina propria extensions into the overlying papillae are well distinguishable, because the papillae are completely fibronectin negative and therefore a high contrast emerges between the green fibronectin and the dark bluish DAPI staining of the cell nuclei.

As for the ki67 positive proliferating cells, they are visualized similarly well in paraffin and cryosections (Fig. 1). Interestingly, adjacent to the lamina propria, there is a cell layer at the margin of the filiform papillae and the taste buds, the so called basal cells, exhibiting many clearly visible dark and ki67 positive cells. Among these cells, progenitor- and stem cell-like cells have been proposed as they show expression of transcription factor p63 that is required for maintenance of self-renewal capacity (Sullivan et al., 2010). In the stroma of the lamina propria, however, practically no proliferating cells are visible. Moreover, the apical extragemmal cells in the cornified cell layers confining the tongue towards the oral cavity also exhibit some amount of ki67 positive cells (Mistretta and Kumari, 2017). In contrast, the rest of the papillae with the taste buds, including fibroblasts and stromal cells, are mostly non-proliferating and have a dark blue, but not brownish-black colour.

Very similar to the previously illustrated fibronectin staining with the fluorescent detection system, paraffin sections have to be preferred over cryosections for α-SMA immunohistochemistry in the healthy rabbit tongue (Fig. 1). Both arteries and veins show α-SMA positive regions in the vessel wall. This is well shown at higher magnifications in the paraffin sections while in the cryosections, it is less clear – also because the green intensity is lower compared with the paraffin sections.

An overview of recommendations to use either formalin fixation or cryopreservation for the antibodies applied in this study can be found in Table 3.

Table 3
Recommendations which technique is favoured for which purpose.

Antibody	*Detection system*	*Recommendation*
Collagen I	Chromogenic	Better distinguishable structures in cryosections caused by higher colour contrast between brown and bluish in contrast to paraffin sections (high brown background).
Collagen III	Chromogenic	At least at higher magnification, collagen III is better visible in paraffin sections, because of overstaining in cryosections, resulting in less distinguishable structures.
Ki67	Chromogenic	Both paraffin sections and cryosections are equally good showing the ki67 positive proliferative cells.
Fibronectin	Fluorescent	Paraffin sections with their high green intensity and clearly confined structures have to be preferred.
α-SMA	Fluorescent	Paraffin sections with their high green intensity and clearly confined structures have to be preferred.

3.2. Antigen retrieval (AR) after formalin fixation and paraffin embedding

As the fixation of tissue in a formalin solution can compromise the immunoreactivity of the antigens, techniques to unmask the epitopes of hidden antigens are daily routine in histopathology. One option is to heat the paraffin sections to approximately 100 °C in a microwave oven (Shi et al., 1991). Also pressure cookers as well as steamers are used, and detailed protocols for AR have been reported (Ramos-Vara, 2017). For

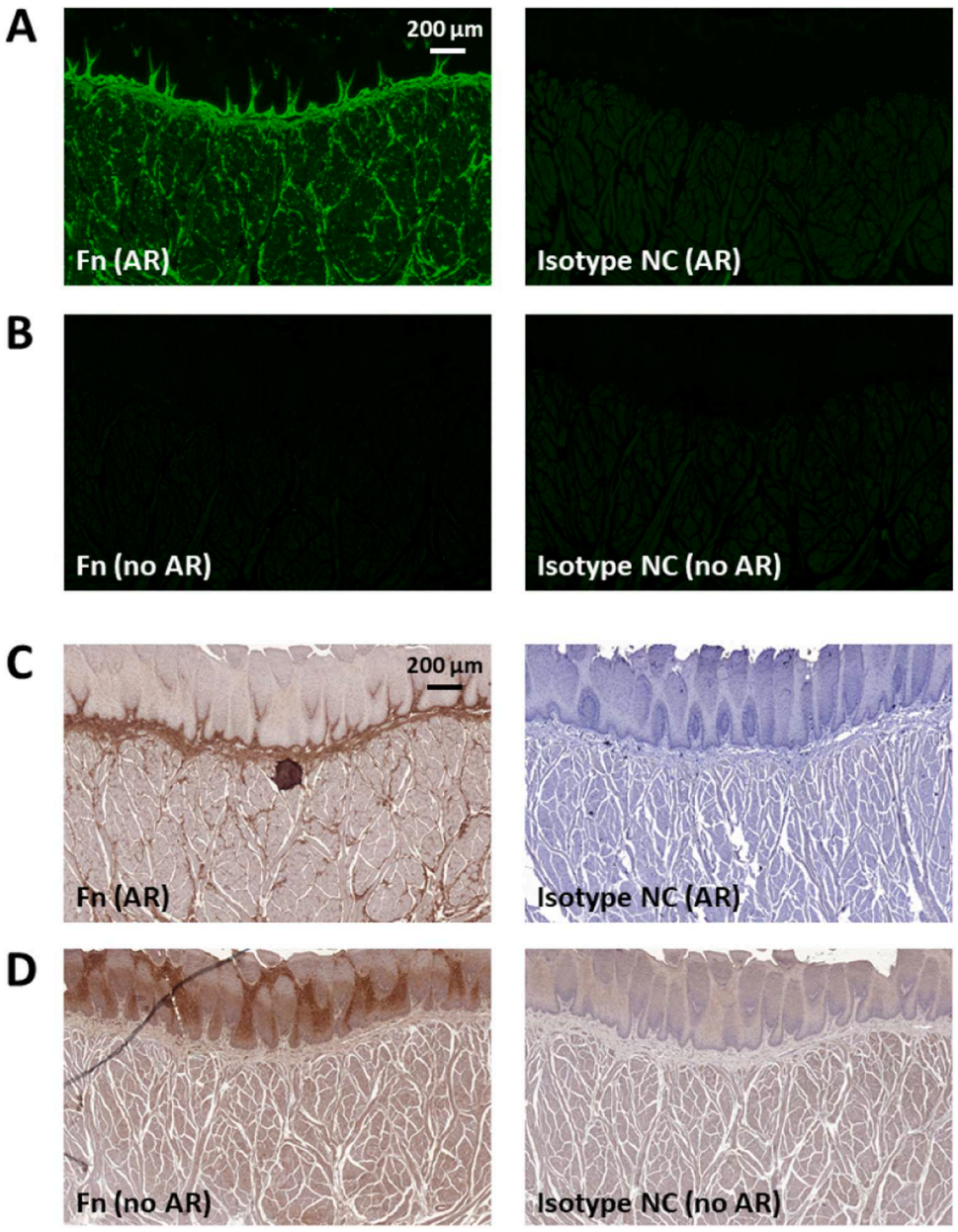

Fig. 2. Comparison of fibronectin immunohistochemical labelling and Isotype negative controls in paraffin sections for fluorescence (A, B) resp. chromogenic stainings (C, D) with antigen retrieval (AR; A and C) or without AR (no AR; B and D) for tongue body tissue.

other fixation techniques that are not crosslinking like formalin, AR techniques have been developed (Stumptner et al., 2019). Importantly, buffers with corresponding pH values and the time for microvave processing have to be adapted and tested for each specific antibody, tissue and and the fixative (Stumptner et al., 2019). Moreover, after heat-induced antigen retrieval proteomics can be applied in order to further analyse the tissue, whereby proteins from paraffin sections are extracted, enabling validation of the immunohistochemical labellings at different locations in the tissue section (Shi et al., 2019).

To show the huge effect of an AR step before immunochistochemical staining in paraffin sections, we have compared fibronectin and isotype negative control labelled sections, with or without AR (Fig. 2). As for the fluoresecent detection system (Fig. 2 AB), the fibronectin positive areas in the rabbit tongue can only be seen when AR was applied. For the chromogenic DAB detection system, fibronectin positive brown colour is distinctive with AR (showing the fibronectin positive endomysium and the lamina propria), however, without AR, there is a quite intense brown overstaining, without clearly showing the fibronectin positive areas (Fig. 2CD). The image from stainings without AR (fibronectin) shows particular brown zones in the filiform papilla, but not in the lamina propria. Correspondingly, the isotype negative control shows a high background staining of light brown colour everywhere in the chromogenic detection system without AR (Fig. 6D). We conclude that for both detection systems (fluorescent and chromogenic) an AR step is favourable in the case of paraffin sections.

3.3. *Fluorescent detection system versus chromogenic staining*

As a next step, we have compared collagen I, collagen III, fibronectin and α-SMA as well as ki67 immunohistochemical labelling in serial sections, for chromogenic DAB / brightfield imaging compared with fluoresecent detection system in paraffin sections with AR (Fig. 3). As can be observed, there is an advantage of the chromogenic system over the fluorescent system for collagen I and III, as well as for fibronectin. On

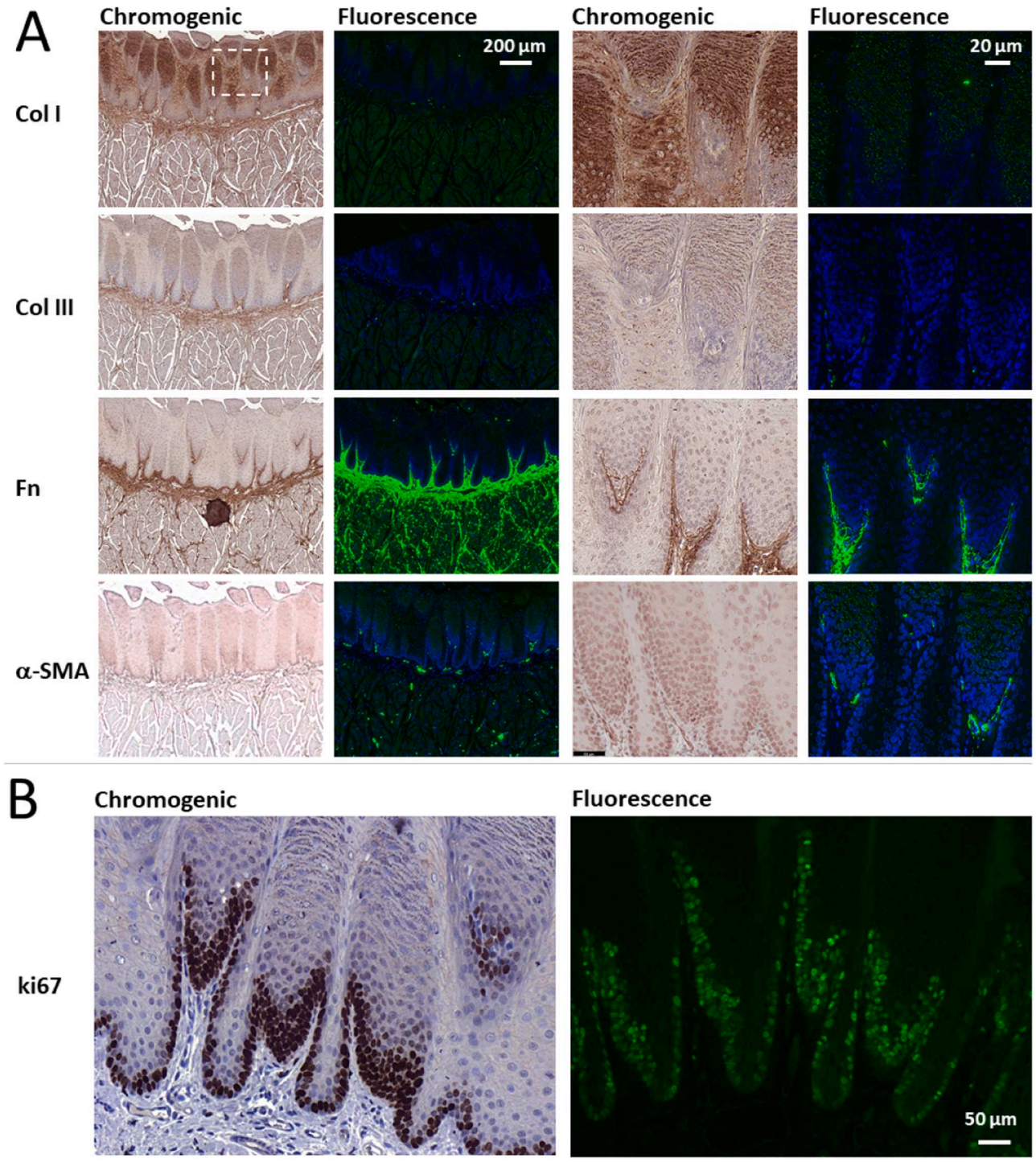

Fig. 3. Immunohistochemical labelling for collagen I, collagen III, fibronectin, a-SMA (A) and ki67 (B) in tongue body tissue, for chromogenic staining with subsequent bright field imaging (left; DAB as chromogen) and immunofluorescence staining and imaging (right) for two magnifications (A). All samples were paraffin embedded and labellings performed with AR.

the other hand, ki67 stainings resulted in equally good images for both systems. The superiority of the chromogenic DAB system is reflected in the distinct appearance of the lamina propria (collagen I and III; fibronectin), while the vessel walls of the veins and arteries are better visible with the fluorescent detection system (α-SMA). As a general consideration when choosing one of these detections systems, optical colour loss in immunofluorescence is a disadvantage over the stable DAB chromogenic system, so in case equally good intensities can be achieved as found for ki67, DAB is recommended rather than fluorescence. Furthermore, it has to be taken into account that while scanning the slides with digital microscopic devices, the slides have to be handled properly and adequately in order to get full information with respect to immunohistochemical labelling of target structures.

3.4. The rabbit tongue tissue – anatomy

The muscular rabbit tongue tissue is furthermore shown in ki67 labelled sections (Fig. 4A), where the vertical muscles are delineated and

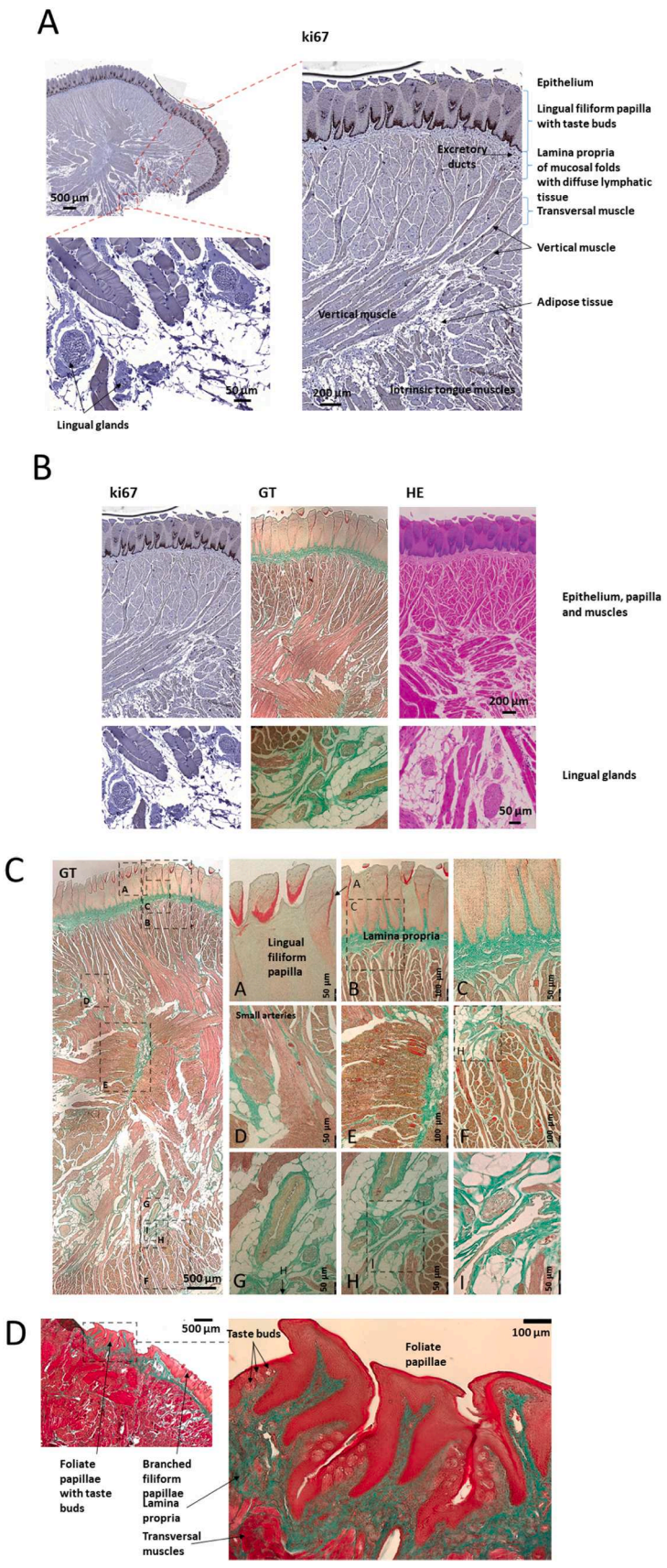

Fig. 4. Specific structures and tissue (depicted with arrows) in a healthy rabbit tongue paraffin section of the body (A, B, and C) and of the lingual prominence large posterior area (D), stained for ki67 with AR, Masson Goldner Trichrom and HE. Overview (A), epithelium, filiform papillae and muscles (B), overview with details in dashed recatangels magnified under A-I (C) and overview with branched filiform and foliate papillae with details of taste buds (D).

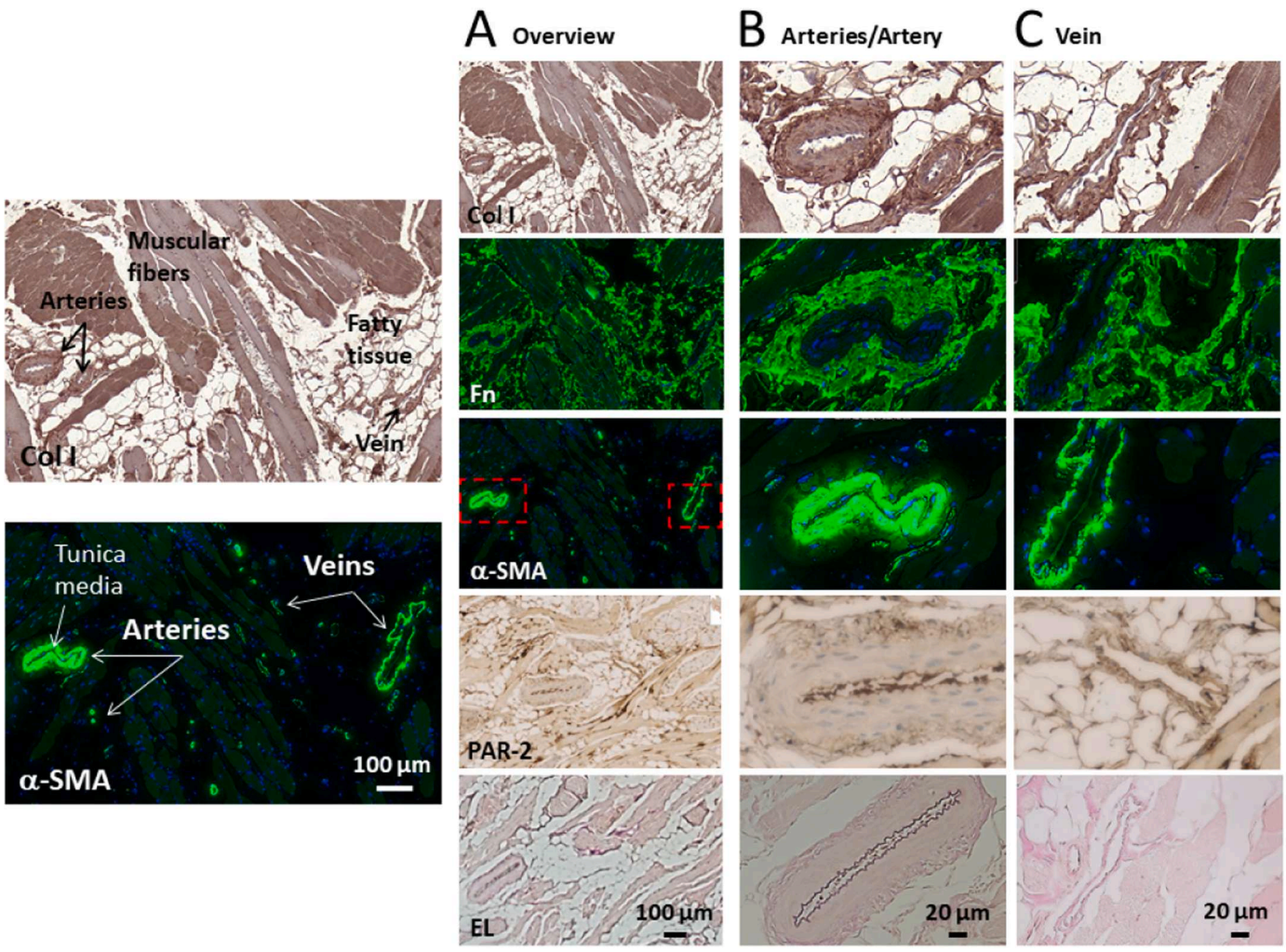

Fig. 5. Arteries, veins, muscular fibers and fatty tissue in rabbit tongue body immunohistochemically labelled for a series of different markers as well as Elastica van Gieson for elastin at different magnifications in paraffin sections. *Key*: Col = Collagen, Fn = Fibronectin, α-SMA = alpha smooth muscle actin, PAR-2 = protease activated receptor-2, EL = Elastica van Gieson staining for elastinAll immunohistochemical stainings were performed with AR.

presented, extruding between the transversal muscles (frontal plane section). Like this, a dense muscular network makes up the intrinsic body of the tongue. Adjacent, the lamina propria and the mucosal folds with diffuse lymphatic tissue as well as the excretory ducts can be seen. Futhermore, the lingual filiform papillae and the strongly proliferating basal cells are presented next to the lamina propria. Although somewhat broken, the epithelium of the tongue is visible as well.

As can be seen in Fig. 4B, the high cell density in the basal region of the lingual filiform papillae was nicely depicted in the HE stained section, where the cells are violet and the stromal tissue and muscle tissue are pink. The collagen rich lamina propria has been visually confirmed by the intense green/turquoise staining in the GT section. Also the lingual glands exhibit a collagen rich wall. This is even better illustrated in Fig. 4C where an overview for a GT stained tongue section is given on the left hand side and some excerpts at higher magnifications are on the right hand side (excerpts A – I in Fig. 4C). Again, the collagen has an intense green/turquoise colour and is situated in the lamina propria (B in 4 C), the small arteries (D in 4 C), and around and between the lingual glands (H and I in 4 C). There were also some regions with an intense red colour in the GT stained sections, like in the detached epithelium (A in 4 C) or the connective stromal tissue (E and F in 4 C), however, we were not able to attribute them to distinct structures or entities. Finally, part of the lingual prominence large posterior area is shown in Fig. 4D. Here, branched filiform papillae and clearly visible taste buds are depicted in a small area of foliate papillae.

Furthermore, we provide collagen I, fibronectin, α-SMA, PAR-2 and EL staining for arteries and veins in overviews in Fig. 5A. Arteries showed a distinct collagen I staining in the vessel wall, while fibronectin protein was not detected in the wall, but adjacent, outside the vessel in the nearby stromal tissue (Fig. 5B). Moreover, α-SMA was strongly expressed in the artery wall. As for the inflammatory marker PAR-2, we found it to be expressed in the endothelium of the arteries, but neither in the *tunica media* nor in the *tunica adventitia* or *tunica intima*. Finally, elastin was expressed in the *tunica intima* of the tongue arteries, circumventing the lumen.

Regarding the veins (Fig. 5C), a quite similar picture was presented as for the arteries, at least for some markers. Collagen I expression was found in the vein wall and fibronectin was situated around the veins, in the stroma. With regard to α-SMA, we found a strong expression in the vein wall. PAR-2 expression on the other hand was rather weak in veins (though not negligible), particularly when compared to the strong expression found in the endothelium of the arteries (Fig. 5B). The most obvious difference, however, was found for the elastin staining, which was completely absent in the veins (Fig. 5C), while in the arteries there was clear lumen confining area of elastin tissue (Fig. 5B). Besides, around arteries as well as around veins, there was some fat tissue, indicated by the large fat droplets these cells contain in their cytoplasm, between the otherwise predominant muscular tissue.

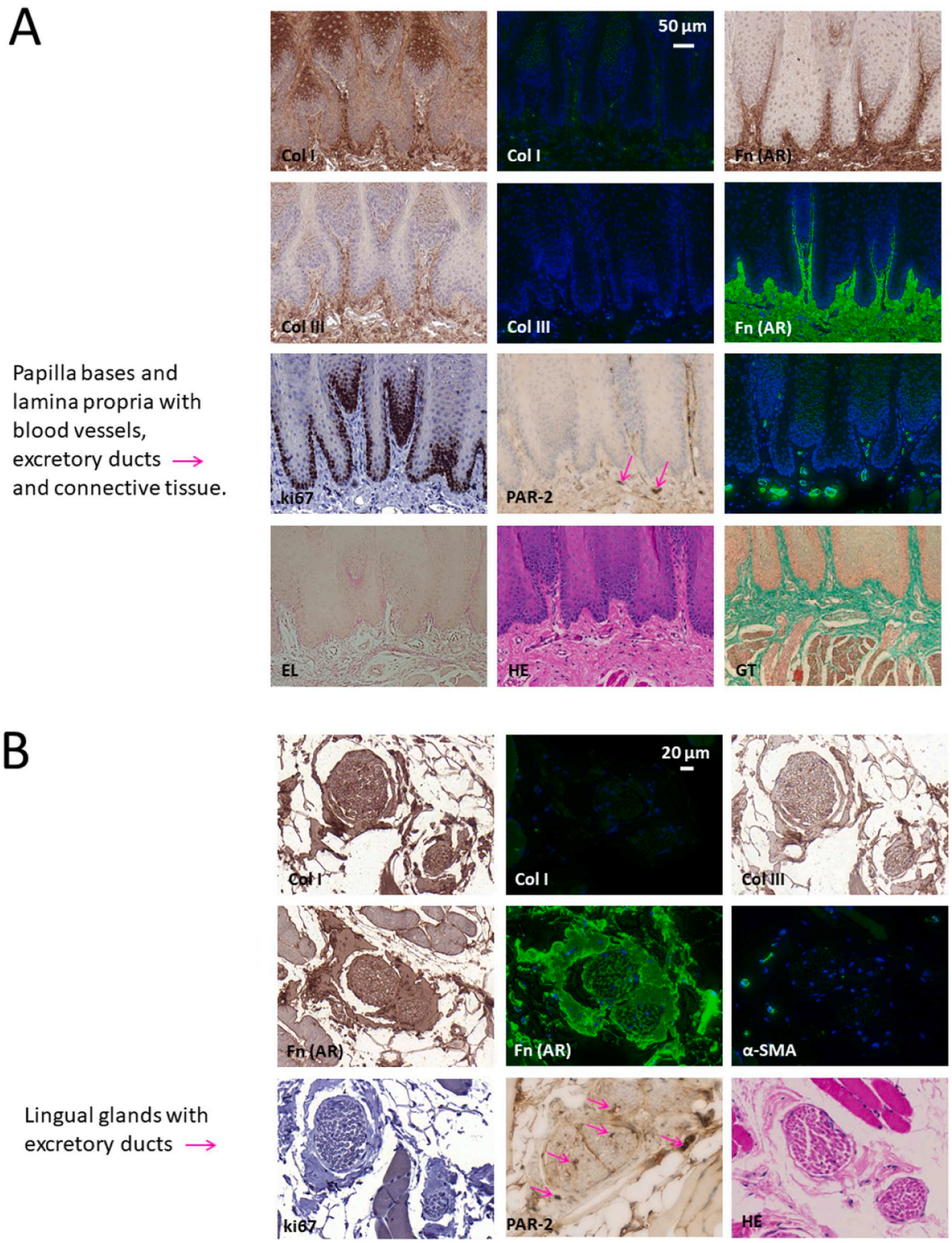

Fig. 6. Papilla bases, lamina propria and excretory ducts (**A**) and lingual glands with excretory ducts (**B**) for paraffin sections of the tongue body stained with collagen I, collagen III, fibronectin, α-SMA, ki67, PAR-2 and HE, respectively. *Key*: Col = Collagen, Fn = Fibronectin α-SMA = alpha smooth muscle actin, ki67 = proliferation marker ki67, PAR-2 = protease activated receptor-2, HE = Haematoxylin&Eosin. All stainings were performed with AR. Excretory ducts are indicated by pink arrows.

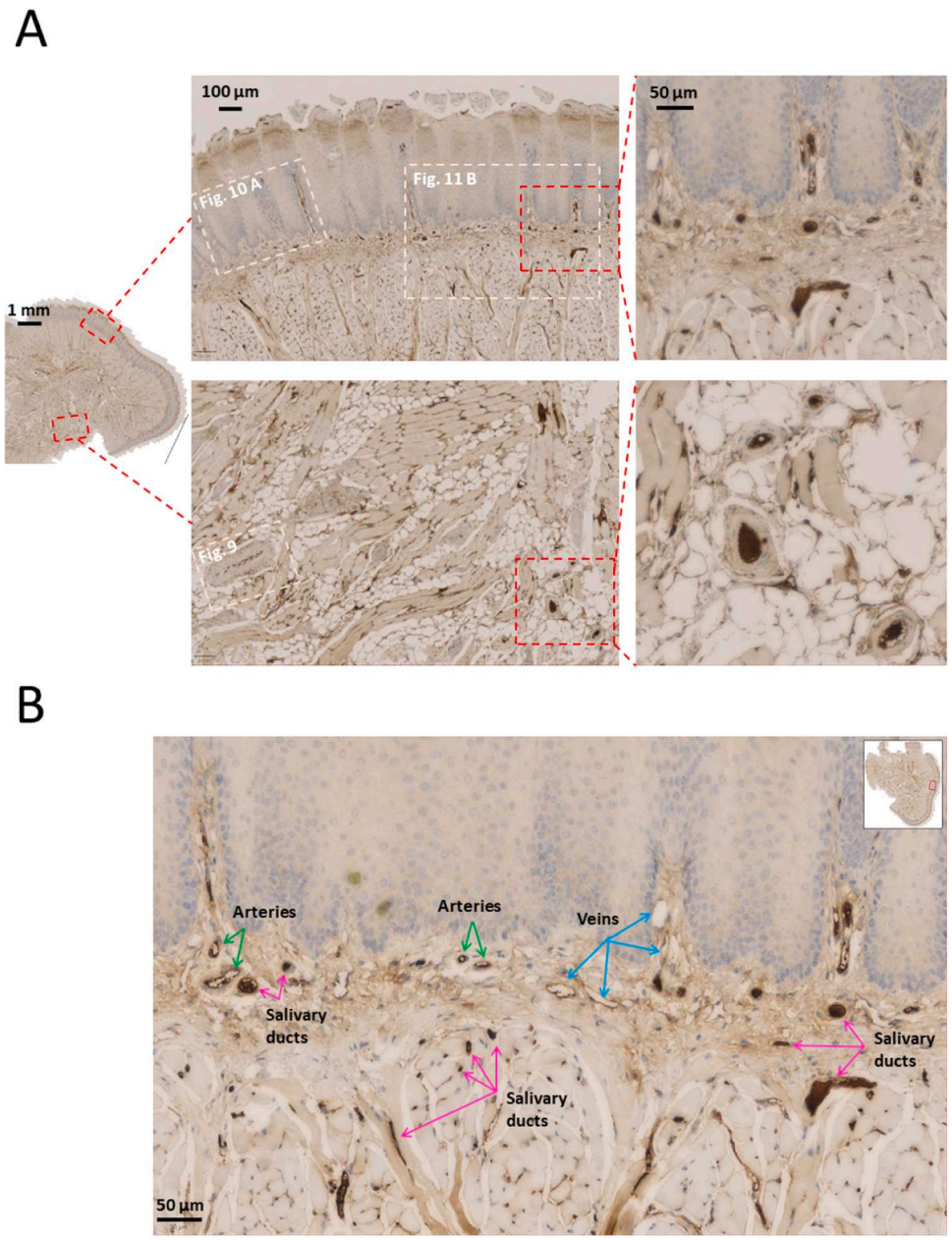

Fig. 7. PAR-2 immunohistochemically labelled paraffin sections of rabbit tongue body tissue. Filiform papilla, lamina propria, muscles and adipose tissue (A) and lamina propria with arteries, veins and salivary ducts (B). Dashed lines confine areas shown in higher magnifications; arteries and salivary ducts exhbit strong PAR-2 staining, shown in lamina propria and connective tissue.

Furthermore, serial sections of papilla bases with blood vessels in the lamina propria are shown in Fig. 6A. A direct comparison of the local distribution of ECM components collagen I and III as well as fibronectin, elastin and α-SMA is possible. While collagen I expression is strong in the filiform papillae and in the lamina propria, collagen III is less prominent, and fibronectin is abundant in the lamina propria, but not in the papillae. Moreover, α-SMA is restricted to vessel walls and some weak expression in the stroma of the papillae. Elastin, however, was not strongly expressed, except for a thin layer in the endothelium of the artery, facing the lumen. Strikingly, the excretory ducts exhibited a strong PAR-2 expression (arrows in Fig. 6A).

A closer look at the lingual glands can be taken at Fig. 6B. While they were collagen III-, α-SMA- and ki67-negative, the glands expressed collagen I and fibronectin. Again, the excretory ducts exhibited a strong PAR-2 expression.

PAR-2 expression for the healthy tongue body has not been examined experimentally so far. Under pathological conditions, however, PAR-2 has been reported to be co-expressed with the transient receptor vanilloid channel 1 (TRPV1) on tongue afferents. Components of spicy food activate TRPV1. Utilizing a conditioned place aversion assay, it has been reported that PAR-2 mediates oral cancer-induced and TRPV1-evoked chemosensitivity in a mouse model (Scheff et al., 2022). Another study, also related to cancer, has reported a PAR-2 dependent mechanism of action for the nociception, contributing to oral pain in squamous cell carcinoma (Tu et al., 2021). Although not a gland of the tongue, the parotid glands in the mouth have furthermore been reported to involve PAR-2 in case of parotid pain (Tu et al., 2021). Due to few literature about PAR-2 expression in the tongue, we have illustrated the PAR-2 positive salivary ducts at higher magnification in Fig. 7A. It can be seen that there is a strong PAR-2 expression in these ducts. In addition, the arteries exhibited PAR-2 expression in the vessel wall, however, relative brown intensity of the PAR-2 staining was stronger in the

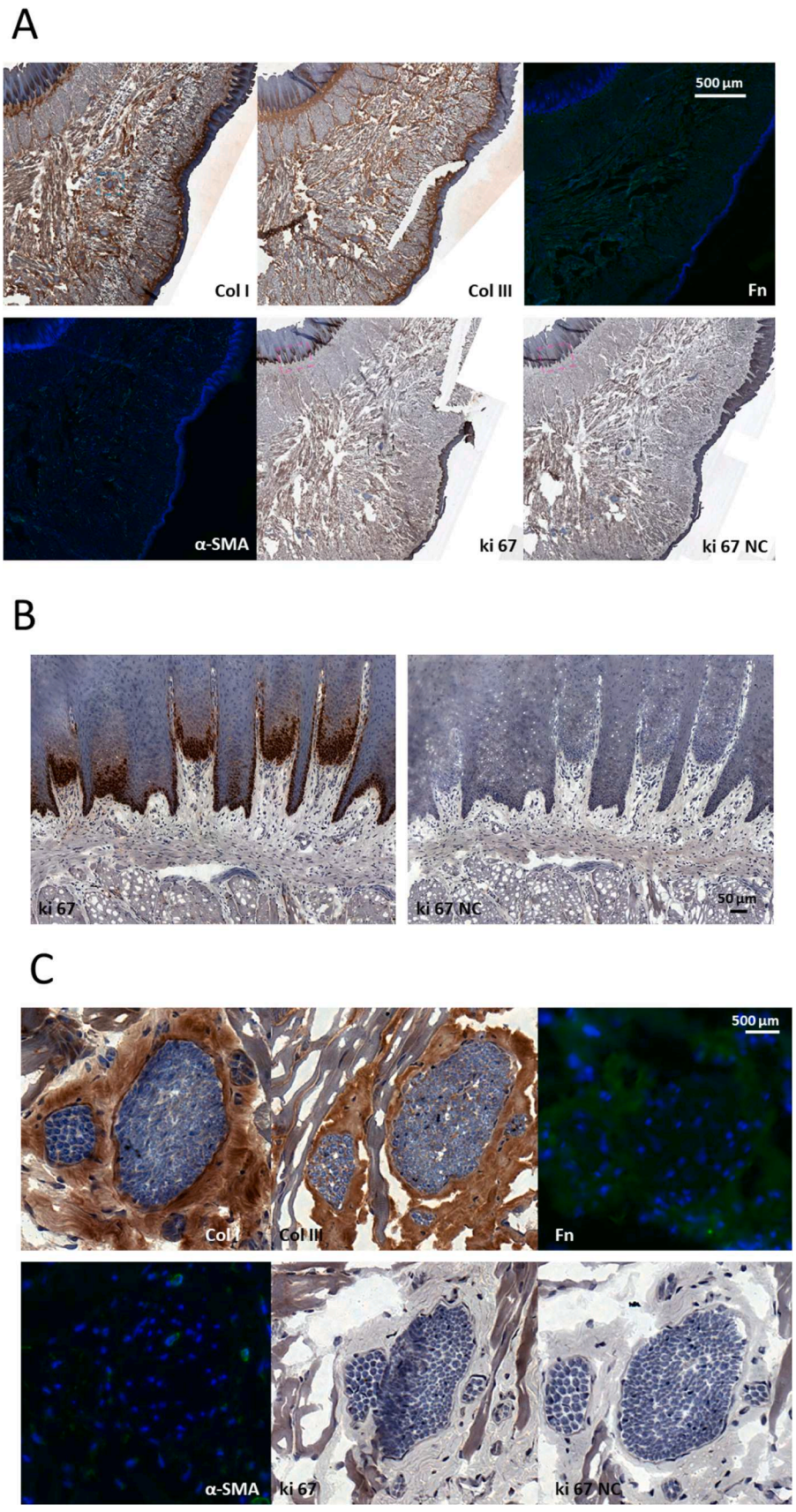

Fig. 8. Cryosections of papilla and muscles (A), magnified areas (B, with areas confined by dashed lines in A) and lingual salivary glands (C) of a healthy rabbit tongue body. Dashed blue line in Col 1 in A confine the areas shown with higher magnifications in B. *Key*: Col = Collagen, Fn = Fibronectin, α-SMA = alpha smooth muscle actin, ki67 = proliferation marker ki67, NC = negative control.

salivary glands compared with the arteries (Fig. 7B).

Finally, in order to demonstrate papilla, muscle tissue and lingual salivary glands also in cryosections, these structures are shown for collagen I, collagen III and fibronectin staining as well as α-SMA and ki67, respectively (Fig. 8). While fibronectin and α-SMA are distinct at low magnifications in Fig. 8A, they are rather blurry in the images of the salivary glands shown at high magnifications in Fig. 8B. Moreover, the salivary glands with their proximate collagen I and collagen III positive areas can be delineated nicely in cryosections (Fig. 8B).

3.5. Conclusive remarks

We have shown that histological staining for elastin, haematoxylin&eosin, Masson Goldner Trichrom and immunohistochemical labelling for collagen I and III, α-SMA, fibronectin, ki67 and PAR-2 is possible in the healthy rabbit tongue tissue and helps to delinate the target structures, such as the salivary glands, the taste buds, lamina propria and the predominating muscle tissue. By comparing paraffin and cryosections, we have given recommendations which kind of fixation and embedding technique might be favoured and give reasons for the specific choice based on the assessed images and their relative magnifications. Moreover, we depicted the clear advantage of an antigen retrieval step for formalin fixation and paraffin embedding. Finally, as PAR-2 may be involved in tongue pain, we depicted the PAR-2 distribution and found a predominant PAR-2 protein expression in the arteries, veins and particulary the salivary ducts of the tongue.

This technical note is limited to the healthy rabbit tongue tissue – mainly of the tongue body – and does not provide images for a diseased state. Depicting different tongue diseases would be beyond the scope of this study. It would not be possible to cover all kinds of diseases without expanding the manuscript too much. Moreover, the tongue body sections were taken from only one rabbit, while the lingual prominence large posterior area were taken from a second rabbit, so potential inter-donor biological variability cannot be addressed. It has to be noted, however, that healthy New Zealand white rabbits are very similar (same genetic background); and their similarity is more pronounced among healthy rabbits than under a pathological situation. As a consequence, one healthy tongue to study the tongue body seems to be enough to delineate this organ for different stainings and immunohistochemical labellings.

To sum up, we provide histological and immunohistochemically stained sections of the healthy rabbit tongue which can be used to be compared to the pathological situation for researchers choosing this animal model.

Author contributions

GMB performed IHC stainings (except for PAR-2), imaged all sections and composed all figures. DMH gave valuable input on PAR-2 staining. IM and JR helped with organ preparation and histology. MC, PG and JB supervised the study. JB provided funding, wrote the manuscript and supervised the study.

CRediT authorship contribution statement

Buschmann Johanna: Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Validation, Writing – original draft, Writing – review & editing. **Giovanoli Pietro:** Supervision, Writing – review & editing. **Calcagni Maurizio:** Supervision, Writing – review & editing. **Rieber Julia:** Data curation, Methodology. **Miescher Iris:** Data curation, Methodology. **Meier Bürgisser Gabriella Meier:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Heuberger Dorothea M.:** Data curation, Methodology, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

Olivera Evrova is highly acknowledged for establishment of IHC stainings (except for PAR-2) and for her comments on the manuscript. We thank Flora Nicholls for tongue extraction from the cadaver rabbit. Furthermore, we thank Ines Kleiber-Schaaf and Andrea Garcete-Bärtschi for their help with tissue sections and HE, GT and EL stainings. We thank Silvia Behnke from Sophistolab, Switzerland, for PAR-2 immunohistochemical staining.

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