

## ORIGINAL RESEARCH

# Vocal fold mucus layer: Comparison of histological protocols for visualization in mice

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**Abstract**

**Objectives:** The epithelial associated mucus layer of vocal fold (VF) mucosa, plays an essential role in protecting and lubricating the tissue, as well as promoting normal voice quality. Serving as a habitat for laryngeal microbiota involved in the regulation of host immunity, VF mucus contributes to laryngeal health and disease. However, its unstable structure renders its investigation challenging. We aim to establish a reproducible histological protocol to recover the natural appearance of the VF mucus layer for investigation.

**Methods:** Using a murine model, we compared the suitability of multiple fixation methods—methacarn, formalin, and cryopreservation followed by post-fixation with formalin, paraformaldehyde (PFA), acetone, and two staining methods—Alcian Blue (pH 2.5)/Periodic Acid Schiff (AB/PAS) or PAS. Fixation and staining outcomes were evaluated based on the preservation of tissue morphology and mucus layer integrity. Mucin proteins, Muc1 and Muc4, were stained to validate the presence of mucus layer overlaying the VF mucosa.

**Results:** Methacarn fixation followed by PAS staining was capable of preserving and displaying the smooth and continuous mucus layer, ensuring the determination of mucus thickness and mucin staining.

**Conclusions:** Our study is the first to establish a histological protocol for the visualization of the in situ VF mucus layer whereby facilitating the study of VF mucus biology including VF surface hydration, ion/nutrients transports, biomechanical properties that maintains normal voice quality as well as VF pathophysiology and host-microbe interactions in the larynx.

**Level of Evidence:** N/A.

**KEYWORDS**

histology, mouse, mucin, mucus layer, vocal fold

## 1 | INTRODUCTION

Vocal fold (VF) mucosa, comprised of epithelium and lamina propria, is covered by a thin layer of mucus which serves to support optimal

biomechanical properties of the VF and promote normal voice quality<sup>1,2</sup> (Figure 1A). Mucus is a heterogenous mixture of more than 95% water in addition to salts, proteins, and lipids.<sup>3</sup> Despite the high water content, mucus possesses many physical properties of a solid,

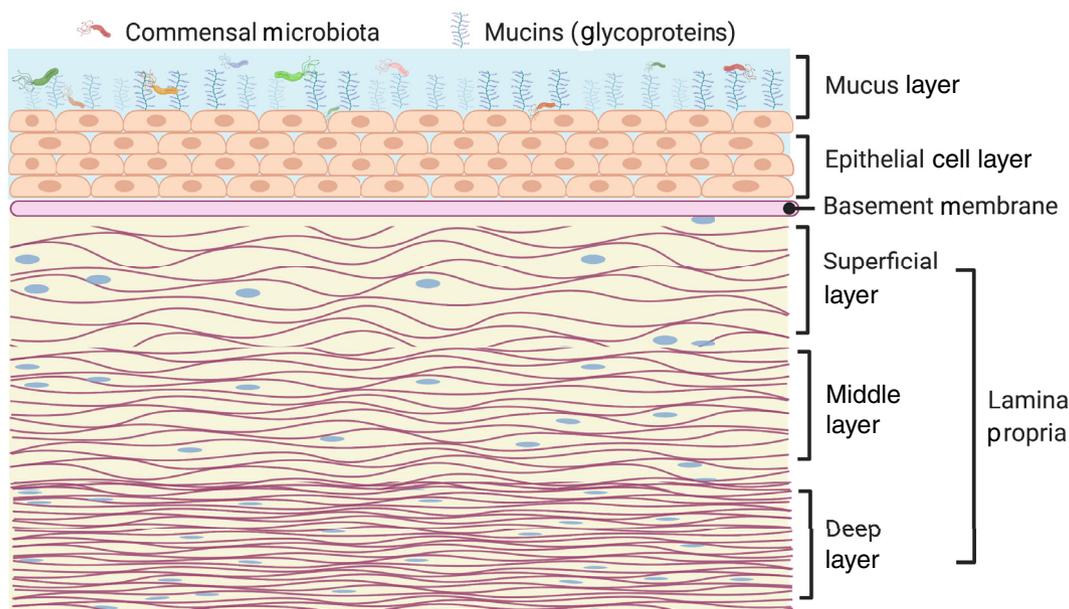
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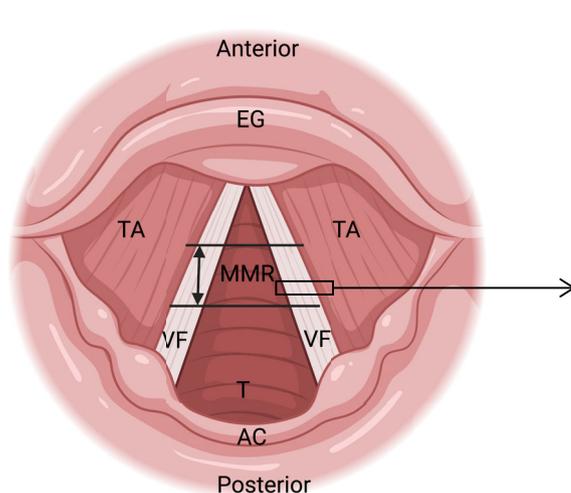
attributed to the presence of various mucins—hydrophilic protein complexes with high number of O-glycosylations.<sup>4</sup> The mucus layer forms an viscoelastic physical barrier that protects the underlying tissue from being exposed to external irritants, such as pathogens, particles, and toxic chemicals in inhaled air and gastroesophageal reflux.<sup>5</sup> It also serves as a natural habitat for commensal microbiota, which can trigger enhanced structural protective features against pathogen invasions, such as faster epithelial cell turnover, increased mucus layer thickness, and greater mucin densities.<sup>4,6–8</sup> Common observations of increased mucus production, as well as viscosity, in patients with

voice disorders may suggest the likelihood of a similar mucous structural reinforcement in VF to regulate host defense or affect VF vibration.<sup>9</sup> It is thus essential to examine the VF mucus layer barrier defense associated with healthy and pathogenic microbial communities to fully interrogate mechanisms of laryngeal pathology to ensure optimal treatment paradigms.

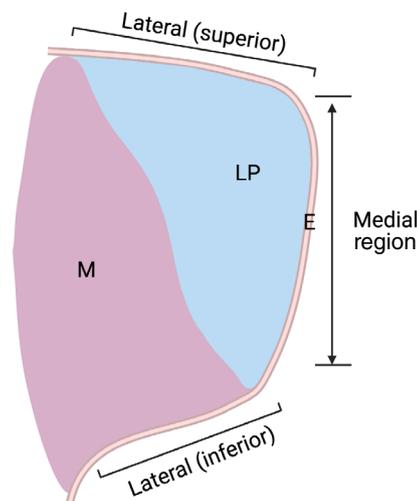
The labile structure of VF mucus, however, renders its histological characterization and quantification challenging from a methodological perspective.<sup>10</sup> Several studies that interrogated the gastrointestinal (GI) mucus layer report that traditional aqueous



(A)



(B)



(C)

**FIGURE 1** A schema of the VF mucus layer and its distribution along the mucosa surface. (A) A thin and gel-like mucus layer covers the VF mucosa. Various commensal bacteria reside in the VF mucus layer. (B) A transverse view of VF showing mid-membranous region, where AC, EG, MMR, T, TA, and VF represents arytenoid cartilage, epiglottis, mid-membranous region, trachea, thyroarytenoid muscle, and vocal fold. (C) Coronal view of VF showing medial and lateral (superior/inferior) orientation where E, LP, and M represents epithelium, lamina propria, and muscle

fixatives such as 10% Neutrally Buffered Formaldehyde (NBF, aka formalin) result in the loss or collapse of the mucus once it comes into contact with water and non-aqueous Methacarn (or Carnoy's) solution, consisting of 60% (vol/vol) absolute methanol (or ethanol), 30% chloroform, and 10% glacial acetic acid is capable of preserving mucus integrity, allowing for the measurement of mucus thickness in human and animal guts.<sup>11-13</sup> While, others suggest instant freezing of tissues in liquid nitrogen or optimal cutting temperature (OCT) compound followed by cryosectioning and post-fixation in formalin, paraformaldehyde (PFA), or sucrose solution to achieve optimal mucus preservation.<sup>14-17</sup> Reports on the mucus layer length and thickness from these studies are often contradictory, and conflicting recommendations has made the selection of a protocol difficult for VF mucus layer investigation. Further, the VF mucus layer of a healthy individual appears to be thinner and looser than that of the gut, possibly due to the frequent vibration as well as unique location devoid of the luminal content as accumulated in the gut,<sup>9</sup> resulting in greater vulnerability to traditional fixatives. Apart from fixation, histological staining methods have a direct impact on the visualization of mucus.<sup>18</sup> While Alcian Blue (AB)/Periodic Acid Schiff (PAS) has been considered the gold standard for mucus staining,<sup>19</sup> numerous modifications have been made to standard protocols, in terms of stains, pH, staining times and order, and so on, depending on tissue type that varies in mucus composition and structure.<sup>14,20</sup> To date, there is a paucity of work optimizing histological visualization of the mucus layer in vocal folds.

The goal of this study is to establish a reproducible protocol for recovering the natural appearance of the VF mucus layer histologically by comparing multiple fixation and staining methods in a murine model.

## 2 | MATERIALS AND METHODS

### 2.1 | Tissue collection

This study was completed in accordance with protocol (M005669) approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison. Twenty-four male 12-week-old C57BL/6J mice were obtained from the Biomedical Research Models Services (BRMS) Mouse Research Breeding Core at the UW-Madison. Animals were euthanized with CO<sub>2</sub> according to BRMS guidelines. Cervical dislocation was not performed after CO<sub>2</sub> to avoid any external pressure that may potentially impair the intactness of VF mucosa. Larynges were removed and fixed immediately as below. Colon was collected as positive control for mucus staining.

### 2.2 | Tissue fixation and sectioning

Larynges were fixed in one of three ways—formalin ( $n = 8$ ), methacarn ( $n = 8$ ) or cryopreserved ( $n = 8$ ; Table 1). For formalin fixed samples, larynges were placed in 1.5 mL microcentrifuge tube filled with formalin, fixed overnight at 4°C, and remained in 70% ethanol until standard

tissue processing procedures for paraffin-sections. Alternatively, tissues were fixed overnight in a freshly made nonaqueous methacarn solution (60% (v/v) methanol, 30% (v/v) chloroform, and 10% (v/v) glacial acetic acid), washed in methanol (2 × 30 min) and 100% ethanol (2 × 20 min), cleared in xylene (2 × 15 min), and infiltrated with hot paraffin (60°C) for 2 hours. Paraffin-embedded tissues were cut coronally ( $n = 4$ ) or transversely ( $n = 4$ ) into 5- $\mu$ m sections and stored at 4°C. For cryopreserved samples, larynges were placed in a cryomold and embedded with OCT compound (Sakura Finetek, Torrance, California) on dry ice. Frozen tissues were kept in -20°C until cryosectioning into 5- $\mu$ m coronal or transverse sections. Cryosections were post-fixed in formalin, 4% PFA, or 100% acetone for 30 minutes.

### 2.3 | Mucus layer staining

Combined AB/PAS or PAS only staining (Newcomer, Middleton, Wisconsin) was performed to visualize mucus layer in paraffin-embedded (formalin/methacarn-fixed) or cryopreserved VF/colon sections, with four serial sections (coronal/transverse) per mouse and four mice per fixation method (Table 1). Paraffin-embedded sections were heated on a slide warmer at 60°C for 20 minutes, deparaffinized in xylene (3 × 3 minutes), hydrated through 100% and 95% ethanol (2 × 10 dips), washed in distilled water (1 minutes). Subsequently, deparaffinized sections were stained with PAS or AB/PAS according to the manufacturer's instructions. Cryosections were removed from -80°C and equilibrated in room temperature for 30 minutes, fixed with formalin, 4% PFA, or acetone, and subject to the same staining procedures as paraffin-embedded sections. Images were taken with a Nikon Eclipse Ti2 inverted microscope (Nikon Instruments Inc., Melville, New York) with a DS-Ri2 high-speed color camera (Nikon Instruments Inc., Melville, New York) and were adjusted for brightness with a corresponding analysis software NIS Elements (Nikon Instruments Inc., Melville, New York).

### 2.4 | Mucin protein staining

Immunofluorescence staining of Muc1 and Muc4 proteins was performed to validate the optimal fixation method determined above. Paraffin-embedded sections (four serial sections per mouse, four mice per cutting orientation) were deparaffinized, rehydrated, and stained using a standard IHC protocol.<sup>21</sup> Antigen retrieval was performed by heating sections in sodium citrate pH = 6 at 80°C water bath for 2 hours. Primary antibodies were anti-Muc1 and anti-Muc4 diluted at 1:100 (both Abcam, Cambridge). Secondary antibody was Cy 3 AffiniPure F(ab')<sub>2</sub> Fragment Goat Anti-Mouse IgG (H + L; Jackson ImmunoResearch Inc., West Grove, Pennsylvania) diluted at 1:200. Sections were incubated with the primary antibodies at 4°C overnight followed with secondary antibodies for 1.5 hours. Slides were mounted using Vectashield with DAPI (Vector Laboratories, Burlingame, California). Cryosections were air-dried for 2 hours, washed

**TABLE 1** Overview of the histological fixation and staining performed on murine VF section

Fixation method	Cutting (# of animals)	AB/PAS staining		PAS staining		Muc1/4 staining
		Staining performed	Mucus length or thickness measurement	Staining performed	Mucus length/thickness measurement	
Formalin	Coronal (4)	✓	✓	x	x	x
	Transverse (4)	✓	✓	x	x	x
Methacarn	Coronal (4)	✓	✓	✓	✓	✓
	Transverse (4)	✓	✓	✓	✓	✓
Cryopreservation	Coronal (4)	✓	x	x	x	x
	Transverse (4)	✓	x	x	x	x

Note: Check mark represents assessed.

with PBS (2 × 5 min), fixed with formalin, PFA, or acetone as mentioned above, then stained with Muc1 or Muc4 following the same protocol above. Images were taken with the Nikon Eclipse Ti2 inverted microscope with a DS-Qi2 high-sensitivity monochrome camera (Nikon Instruments Inc., Melville, New York) and adjusted for brightness using the NIS Elements software.

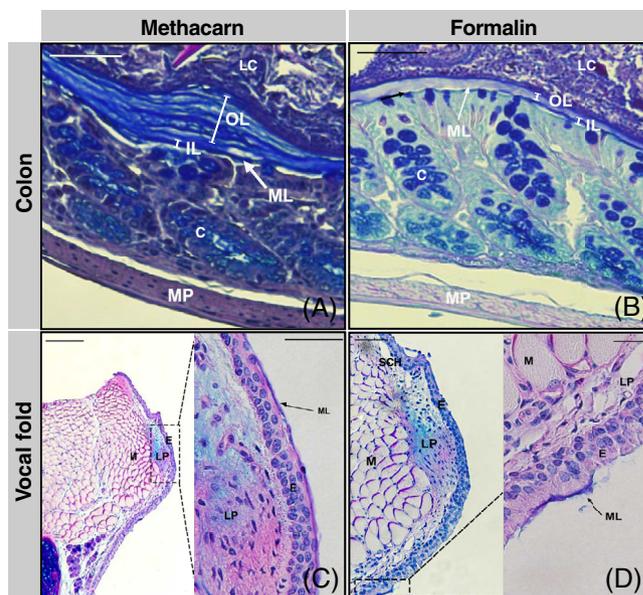
## 2.5 | Mucus layer thickness measurement

Mucus layer thickness was determined in AB/PAS- or PAS-stained VF/colon sections by measuring three mucus fragments per section, four serial sections per mouse, four mice per staining method. Mucus fragments longer than 300 μm were considered continuous and were measured for thickness. At least four thickness measurements were performed per fragment with the measuring tool embed in the NIS element software. Thickness was defined as the distance from the apical surface of epithelial layer to luminal surface. Continuous mucus layers longer than 300 μm were measured and only the length of the longest mucus layer was reported for each animal.

## 2.6 | Assessment of fixation and staining efficacy

Fixation efficacy was assessed for AB/PAS-stained VF coronal sections by examining 16 sections per fixation method—four serial sections per mouse, four mice as biological replicates. Efficacy was primarily determined by the ability to preserve the integrity of mucus layer and the underlying mucosa. Given the dramatic structural and compositional differences between the mucus layer and mucosa, these were rated separately based on multiple criteria. Specifically, mucus integrity rating was based on the estimated percentage of mucus layer occurrence, continuity (>300 μm), and thickness (>7 μm); mucosa integrity rating was based on the estimated percentage of epithelial integrity and visualization of subcellular structures (nucleus and cytoplasm). The sum of the percentages was used as final score for mucus layer and mucosa, respectively.

Staining efficacy was assessed for AB/PAS- or PAS-stained vocal fold sections fixed with the optimal method determined. Sixteen



**FIGURE 2** Comparison of mucus fixation methods between paraffin-embedded sections. (A,B) Colon tissue fixed in methacarn or formalin, respectively; (C,D) VF tissue fixed in methacarn or formalin, respectively. C, E, IL, LC, LP, M, ML, MP, OL, and SCH represents crypts, epithelium, inner layer, luminal contents, lamina propria, muscle, mucus layer, muscularis propria, outer layer, and swiss-cheese effects, respectively. Black arrows denote the gel-like mucus layer overlaying the mucosa surface. Scale bars in the panel of A (left), B (left), C, D = 500 μm; scale bar in A (right) = 100 μm; scale bar in B (right) = 50 μm

sections (four section per mouse, four mice as biological replicates) were selected per staining method for comparison, with at least one continuous (>300 μm) mucus fragment present per section regardless of cutting orientation. Staining efficacy was rated with respect to the estimated percentages of mucus continuity, thickness, as well as the ability to show mucin types (Muc1 and Muc4) and their distributions. A final score, which is the sum of the estimated percentages, was assigned to each method to indicate the overall efficacy. Two raters were employed for blinded review of the tissue sections. Each experiment was performed twice to ensure reliability.

TABLE 2 Comparison of fixation efficacies between various fixation methods

Fixatives	Mucus layer				Underlying mucosa					Scores
	% of sections with mucus fragment(s)	% of sections with continuous mucus fragment(s)	% of sections with >3 continuous mucus fragments	% of sections with mucus thickness >7 $\mu\text{m}$	% of sections with >90% intact epithelium	% of sections without deformed epithelium	% of sections without swiss-cheese holes	% of sections able to display subcellular localization	Scores	
Formalin	0.30	0.00	0.00	0.30	0.88	1.00	0.75	1.00	3.63	
Methacarn	0.75	0.50	0.25	0.50	0.88	1.00	0.30	1.00	3.18	
Frozen-formalin	0.06	0.00	0.00	0.00	0.13	0.00	0.00	0.00	0.13	
Frozen-PFA	0.30	0.25	0.13	0.00	0.19	0.00	0.00	0.00	0.19	
Frozen-acetone	0.25	0.25	0.06	0.00	0.25	0.00	0.00	0.00	0.25	

Note: Evaluation was based on the examination of 16 sections from four VF tissue samples.

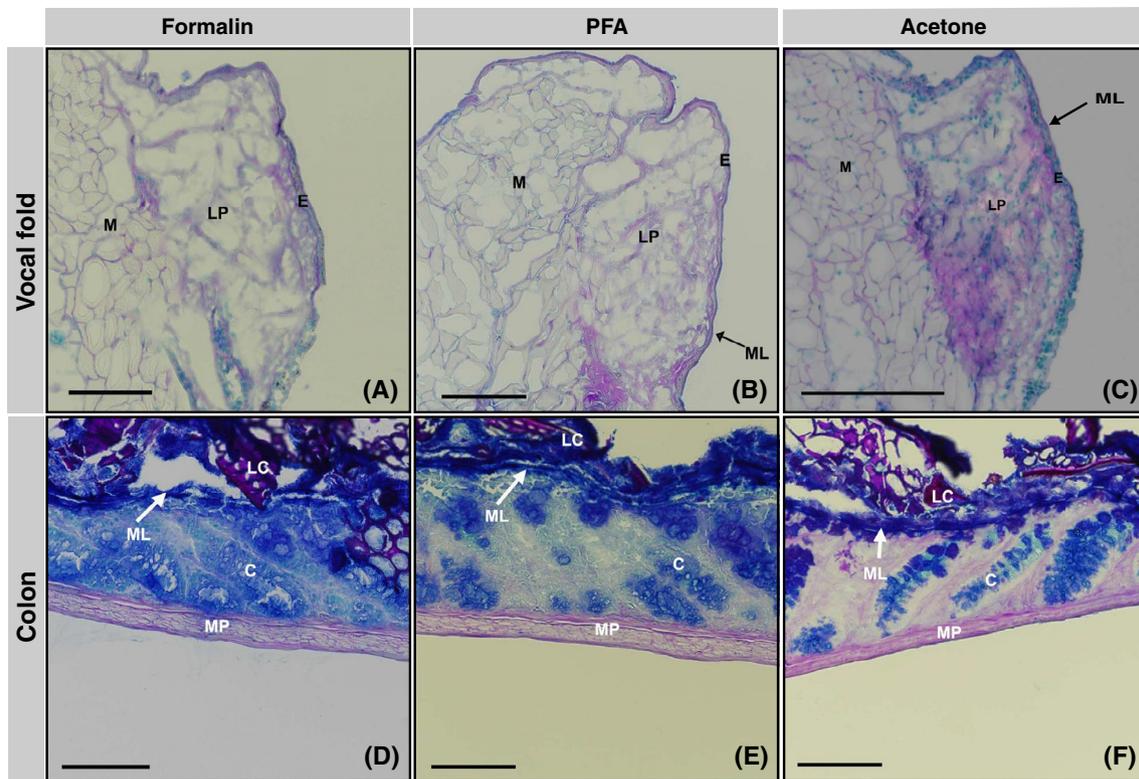
## 2.7 | Statistical analysis

Mean mucus layer thickness for each mouse was determined by averaging the values from at least 48 measurements in four serial sections, three mucus fragments per section, at least four measurements per fragment; mean mucus thickness for PAS or AB/PAS staining method was determined by averaging the values of the mean mucus thickness of four mice. One-way analysis of variance (ANOVA) was performed to compare the mean mucus layer thickness between two cutting orientations and two staining methods, followed by Bartlett's test for homogeneity of variance. Significance was set at  $P = .05$ .

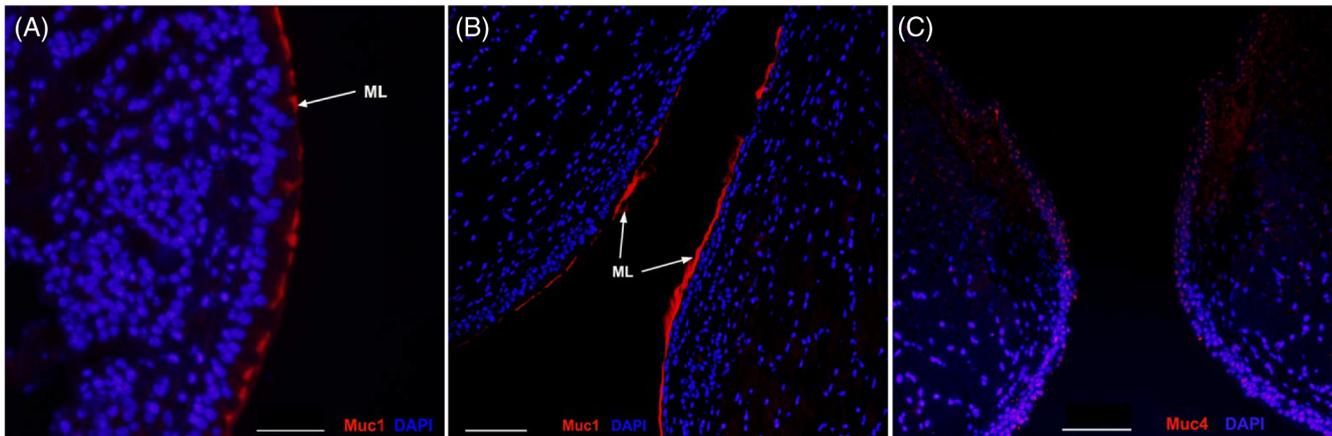
## 3 | RESULTS

### 3.1 | Comparison of mucus fixation methods

The regions of interest in VF tissues, that is, lateral and medial sides of the mid-membranous region, are shown in Figure 1B,C. To determine the optimal fixation method for mucus preservation, AB/PAS staining was employed to visualize the mucus layer in VF tissues fixed in formalin, methacarn, or cryopreserved and post fixed in formalin, PFA, or acetone. Colon tissues, reported to have two distinct mucus layers—stratified thin inner layer devoid of bacteria and thick outer layer rich in bacteria, were included as positive controls to prove the effectiveness of the mucus fixation methods. The colon double-layer mucus was evident in paraffin-embedded sections, where the inner layer could be easily distinguished from the outer layer (Figure 2A,B). However, the mucus layer was indistinct and scattered to some extent in frozen sections; the two mucus layers seemed indistinct and the boundaries between mucus layer and luminal contents/crypts blurred. In regions with no luminal contents, the layer structure was destroyed to varying extents or completely missing in some cases. Methacarn scored highest on mucus preserving capacity in VF tissues, although the mucus thickness was dramatically decreased compared with that in colon, as would be expected (Figure 2C,D, Table 2). VF mucus layer structure was observed in 70% of the sections examined, 50% of the sections retained continuous (>300  $\mu\text{m}$ ) mucus fragment(s) with thickness >7  $\mu\text{m}$ , 25% of them retained more than three continuous mucus fragments. In comparison, no continuous mucus fragments were observed in formalin fixed tissues, regardless of paraffin-embedded or frozen sections. Mucus layer was relatively short and fragmented in formalin fixed tissues and more frequently found in inferior lateral region located next to submucosa glands (Figure 2D). VF mucosa integrity was preserved comparably well in formalin/methacarn according to the criteria in this study—88% of the sections retained intact mucosal epithelium without rupture or deformation, subcellular structures (nucleus and cytoplasm) were evident throughout the sections examined, although



**FIGURE 3** Comparison of mucus fixation methods between post-fixed frozen sections. (A-C) cryopreserved VF tissue post-fixed in formalin, 4% PFA, or acetone; (D-F) cryopreserved colon tissue post-fixed in formalin, 4% PFA, or acetone. C, E, LC, LP, M, ML, and MP represents crypts, epithelium, luminal contents, lamina propria, muscle, mucus layer, muscularis propria respectively. Black arrows show the gel-like mucus layer overlaying the mucosa surface. Scale bars in A to F = 500  $\mu$ m



**FIGURE 4** Immunostaining of mucin proteins in methacarn fixed VF sections. (A) Muc1 protein (red) embedded at the apical surface of the VF top epithelial cell layer; (B) Muc1 protein forming a gel-like layer along the mucosa surface; (C) Sporadic distribution of Muc4 (red) protein in the VF. Mucin proteins were counterstained with DAPI (blue) that stains nuclei. ML represents mucus layer; arrow shows the gel-like mucin layer covering VF epithelium. Scale bars in A to C = 500  $\mu$ m

the frequency of swiss-cheese holes were slightly increased in methacarn fixed tissues.

VF mucosa integrity was poorly preserved in frozen sections (Figure 3A-C). Mucosal epithelium was intact in less than 30% of sections

regardless of the post-fixation method, either ruptured or deformed, with high frequency of swiss-cheese hole artifacts in lamina propria due to the tissue shrinkage (Table 2). Mucus fixation efficacies of PFA and acetone were remarkably higher than that of formalin. Although mucus layer

structure was observed in frozen sections, their continuity or thickness was not as delineated as that in paraffin-embedded sections.

### 3.2 | Immunostaining of mucins in VF tissues

To further demonstrate the presence of mucus layer in methacarn fixed VF tissues, immunostaining of Muc1 and Muc4, two common laryngeal mucins, was performed. Muc1 protein was shown either embedded at the apical surface of the top epithelial cell layer (Figure 4A) or as smooth and continuous layer covering the underlying tissue (Figure 4B). Muc4 did not form a continuous layer but was sporadically distributed throughout the epithelial layers of medial VF region (Figure 4C).

**TABLE 3** Comparison of mucus staining methods between AB/PAS and PAS staining

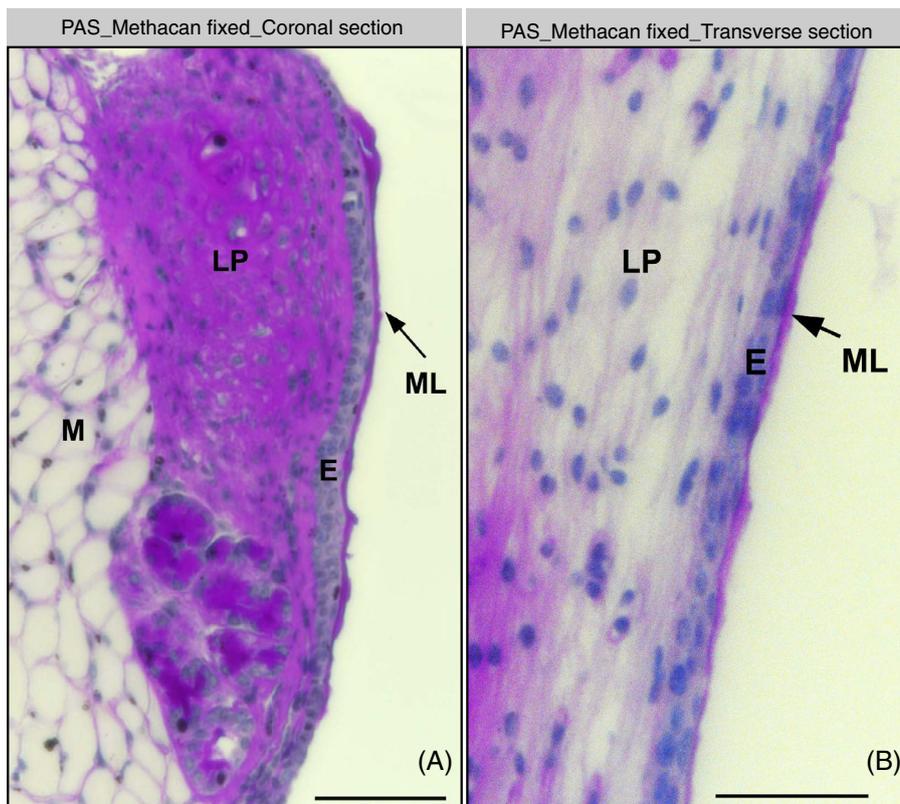
Evaluation criteria	AB/PAS	PAS
% of sections with continuous mucus fragment(s)	0.5	1
% of sections with mucus thickness >20 $\mu\text{m}$	0	0.75
Mucin types and distribution (yes = 1, no = 0)	1	0
Score	1.5	1.75

Note: Evaluation was based on 16 sections with at least one continuous mucus fragment per section.

### 3.3 | Comparison of mucus staining methods

The efficacy of mucus staining method was assessed in VF tissues fixed with the optimal fixative, methacarn, determined previously. The combined AB/PAS staining and PAS only staining were performed and compared with respect to their abilities to display mucus layer in VF. A scoring system was employed, where the efficacy was primarily rated on mucus continuity/length, thickness, as well as the ability to distinguish mucin types and their distribution (Table 3). Interestingly, continuous mucus fragments were observed in all PAS-stained sections evaluated, mainly covering the mucosa lateral surface, while only half of the sections stained with AB/PAS showed continuous mucus fragments (Figure 5). The length of mucus fragment in PAS-stained VF ranged from 2083.66 to 2720.71  $\mu\text{m}$  in coronal sections and 1749.83 to 3606.05  $\mu\text{m}$  in transverse sections, while it was 385.79 to 559.26  $\mu\text{m}$  in AB/PAS-stained coronal sections (Table 4). While the longest continuous mucus layer, 3606.05  $\mu\text{m}$ , was observed in transverse section, mucus layers in transverse sections were on average shorter and less continuous than that in coronal sections. For instance, the mucus fragments in AB/PAS-stained transverse sections were shorter than 300  $\mu\text{m}$  and therefore not considered as continuous, not measured for length or thickness.

Mucus fragment thickness was significantly higher in PAS-stained sections, as compared with AB/PAS-stained sections (Table 3). About 75% of the PAS-stained sections retained mucus fragments with thickness >20  $\mu\text{m}$ , and the mean mucus layer thickness was 41.53  $\mu\text{m}$  for coronal sections and 35.28 for transverse sections (Tables 3 and 4). No significant difference was observed in mucus layer thickness



**FIGURE 5** PAS staining of the mucus layer in vocal fold tissue fixed in methacarn solution. (A) Coronal section; (B) transverse section. E, LP, M, ML represents epithelium, lamina propria, muscle, and mucus layer. The arrows indicate the presence of mucus layer. Scale bars in A and B = 500  $\mu\text{m}$

**TABLE 4** Measurements of mucus layer length and thickness in PAS or AB/PAS stained VF sections

Staining method	Animal ID	Mucus layer length ( $\mu\text{m}$ )	Mucus layer thickness ( $\mu\text{m}$ )				
			Mean	SD	Range	Mean <sup>a</sup>	SD <sup>a</sup>
PAS (coronal)	1	2608.42	33.00	16.08	17.28-80.48	41.53	7.34
	2	2492.05	38.55	13.53	19.43-70.26		
	3	2720.71	44.68	18.32	19.13-85.59		
	4	2083.66	49.90	19.01	21.20-92.64		
PAS (transverse)	1	1749.83	29.26	6.04	18.04-43.40	35.28	5.84
	2	3606.05	32.08	5.62	21.70-45.39		
	3	1809.12	37.28	5.95	26.04-48.02		
	4	1993.10	42.49	6.33	31.04-55.53		
AB/PAS (coronal)	1	487.75	6.49	3.15	2.94-10.45	7.53 <sup>b</sup>	0.78
	2	559.26	7.58	3.37	3.58-9.26		
	3	456.87	8.35	4.25	2.61-10.30		
	4	385.79	7.71	3.72	3.01-11.37		

<sup>a</sup>Indicates the mean mucus thickness/standard deviation of the four biological replicates used in the study.

<sup>b</sup>Denotes mucus layer in AB/PAS stained section is significantly thinner than that in PAS stained section,  $P < .05$ .

between the two cutting orientations, suggesting the mucus layer visualization may not be an impact factor to the mucus layer thickness. In comparison, mucus fragment thickness in AB/PAS-stained sections was  $<20 \mu\text{m}$ , with the mean mucus layer thickness being  $7.53 \mu\text{m}$ .

As expected, AB/PAS staining performed superiorly in displaying the mucin types (acidic mucins stained blue, neutral mucins stained magenta) and their distributions in the VF mucosa and mucus layer (Figure 2A). Various colors in AB/PAS-stained sections could be an indication of multiple types of mucins distributed along the mucus layer and across the VF mucosa. In the epithelial layer, cell nuclei (pale blue) and cytoplasm (light violet) could be distinguished easily with discernable cell boundaries. The basement membrane was stained more magenta, lying right under the epithelial layer, which was not evident in PAS only staining. The turquoise blue found extensively in the upper lamina propria (toward superior VF) could be the representation of sialomucins—a type of acid mucin produced by mucosal glands in small intestine,<sup>22</sup> mesenchymal mucins, or a mix of intracellular mucins and glycogen deposits. The inferior lamina propria region (magenta) could be either neutral mucins, or glycogen and glycoproteins stained positive with alcian blue, or a mix of the two. A combination of these colors, that is, violet/purple, was observed in the transition zone of the two regions, suggesting the mixed presence of the acid and neutral mucins as well as cellular glycogen deposits. In comparison, mucins, basement membranes, and intracellular glycogens storage were collectively stained dark magenta in the PAS only staining, making it impossible to differentiate mucin types or their distribution in mucus layer or mucosa (Figure 5). Taken together, PAS only staining was superior in visualizing the layer structure of the VF mucus in the case where mucin type or distribution was not considered.

## 4 | DISCUSSION

In this study, we established a comprehensive histological pipeline to denote the natural appearance of the VF mucus layer in histological sections, allowing for reliable determination of mucus layer appearance in the murine VF. This is the first attempt to depict the thin layer of mucus in murine VF tissue, aiming to provide methodological guidance to future research in laryngeal research. Our results suggest that both tissue fixation and staining methods have fundamental impacts on the visualization of VF mucus layer. In comparison to formalin, methacarn fixation preserved the smooth appearance of the VF mucus layer to a greater extent with superior reproducibility (Figure 2A, Table 2), stabilizing the mucus structure during fixation with retention throughout subsequent processing. We also found that PAS only staining is superior to combined AB/PAS staining in visualizing the mucus layer on the VF, regardless of its' lack of the ability to distinguish mucin types.

While methacarn was determined to provide the best fixation, none of the fixation methods were absolute in preserving the mucus layer (Table 2). Mucus loss is inevitable, especially for paraffin-embedded sections, where approximately 30% of tissue shrinkage imposed by the ethanol dehydration is expected with downstream processing.<sup>14</sup> Sectioning of the tissue, is also likely to exert additional force which compresses and distorts the mucus layer.<sup>23</sup> Successful applications of formalin in mucus layer preservation have been reported previously in the gut.<sup>24,25</sup> In our study, the mucus layer overlying the VF mucosa was almost completely lost in formalin fixed sections (Figure 2B, Table 2); only mucus fragments near the submucosal glands were retained, indicating that hydration in aqueous fixatives and subsequent paraffin-embedding processes do indeed have adverse impact on mucus layer integrity.

The VF mucus layer is thin relative to that in the colon, intestine, and stomach.<sup>26</sup> This could largely be due to VF mucosa being devoid of luminal contents that remarkably reduces the exposure to hydration and shear forces during processing. Stratified squamous epithelial cells that the mucus layer sits on are not ciliated and therefore less capable of holding water and other mucus contents firmly onto its surface as compared with the ciliated gut.<sup>5</sup> Previous study suggests that the mucus layer in methacarn fixed tissues is generally thinner than that in explanted tissues,<sup>12</sup> suggesting the in vitro VF mucus layer is likely to be thicker in vivo, than our in vitro observations. Indeed, preservation of a continuous mucus covering the entire mucosa seems to be of greater significance than its overall thickness to protecting and lubricating the VF mucosa surface.

Successful preservation of a continuous VF mucus layer is equally essential for the host-microbe interaction studies in the larynx, where investigation of the bacterial localization and host cell immunofluorescence is necessary. The 16S rRNA gene fluorescence in situ hybridization (FISH) has been applied successfully to methacarn fixed tissue from mouse GI tract and shown the spatial organization of gut mucosal bacteria.<sup>27</sup> While these are not yet been assayed in the thin VF mucus layer, we confirmed the feasibility of the host cell staining (mucin staining) in methacarn. While both Muc1 and Muc4 are transmembrane mucins, in the present study, Muc1 was expressed only in epithelial cells on the luminal surface consistent with the observations in human VF.<sup>28,29</sup> The extracellular domain of Muc1 contains a sea urchin sperm protein, enterokinase and agrin (SEA) domain that can be autoproteolytically cleaved in the endoplasmic reticulum which releases the remaining large extracellular domain into extracellular space.<sup>30</sup> The shedding of the Muc1 extracellular domain off the epithelial surface may account for the dense and continuous layer structure on top of the epithelial surface that was measured. Whereas, human ortholog Muc4 is expressed more superficially in human VF mucosa and has been measured in the basal cell layer in a human induced pluripotent stem cell-derived 3D vocal fold model.<sup>29</sup> The sporadic but even distribution of Muc4 within epithelial layers may be a species-specific feature in the mouse. Future research is warranted.

Histochemically, mucins are classified into acid and neutral mucins.<sup>31</sup> Acid mucins are mainly non-sulfated sialomucins (with sialic acid) found in epithelium. Further, a simple-mesenchymal acid mucin that contains hyaluronic acid is present in tissue stroma and sarcomas. Both are positive for AB at pH 2.5 but negative for PAS. Neutral mucins are mostly found in the GI tract, being positive for PAS but negative for AB. We observed the presence of both types of mucins in the mouse VF mucosa, with sialomucin being the dominant acid mucin. In this regard, AB/PAS staining was superior to the PAS only staining, where the distributions of each type of mucins were demonstrated clearly at a glance. This staining technique has been applied to staining of mucus expressed on the apical surface of the epithelial cells in human false VF,<sup>5</sup> where no gel-like mucus layer was observed however, fixation method was not mentioned. The thinner mucus layer observed in with our AB/PAS staining relative to PAS staining could be due to the unequal staining time for periodic acid and Schiff reagent recommended in the manufacturer's instructions; exposure

time to these reagents was reduced by half in AB/PAS staining (Figure 2A). This may result in insufficient staining of the neutral mucins and a thinner mucus layer overlying the VF mucosa, which could be resolved by further optimization extending the staining time for these essential reagents. Knowledge on the VF mucus composition and distribution is limited.

## 5 | CONCLUSIONS

The present study contributes to the establishment of a histological protocol for the visualization of the in situ VF mucus layer in a murine model. Our results demonstrate that non-aqueous methacarn fixation followed by PAS staining produces reliable preservation of the mucus layer with measurable length and thickness. Establishing such methods will allow for foundational investigations for the role of the VF mucus layer in laryngeal health and disease.

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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.

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