Quantification of increased MUC5AC expression in airway mucus of smoker using an automated image-based approach

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Abstract

Microscopic analysis of mucus quantity and composition is crucial in research and diagnostics on muco-obstructive diseases. Currently used image-based methods are unable to extract concrete numeric values of mucosal proteins, especially on the expression of the key mucosal proteins MUC5AC and MUC5B. Since their levels increase under pathologic conditions such as extensive exposure to cigarette smoke, it is imperative to quantify them to improve treatment strategies of pulmonary diseases. This study presents a simple, image-based, and high-processing computational method that allows determining the ratio of MUC5AC and MUC5B within the overall airway mucus while providing information on their spatial distribution. The presented pipeline was optimized for automated downstream analysis using a combination of bright field and immunofluorescence imaging suitable for tracheal and bronchial tissue samples, and air–liquid interface (ALI) cell cultures. To validate our approach, we compared tracheal tissue and ALI cell cultures of isolated primary normal human bronchial epithelial cells derived from smokers and nonsmokers. Our data indicated 18-fold higher levels of MUC5AC in submucosal glands of smokers covering about 8% of mucosal areas compared to <1% in nonsmoking individuals, confirming results of previous studies. We further identified a subpopulation of nonsmokers with slightly elevated glandular MUC5AC levels suggesting moderate exposure to second-hand smoke or fine particulate air pollution. Overall, this study demonstrates a novel, user-friendly and freely available tool for digital pathology and the analysis of therapeutic interventions tested in ALI cell cultures.

KEYWORDS
air–liquid interface, automated quantification, cell profiler, image-based quantification, MUC5B, mucin, smoking

1 | INTRODUCTION

Computational-assisted microscopy advanced remarkably in recent years covering numerous biological and medical research fields (Eggerschwiler, Canepa, Pape, Casanova, & Cinelli, 2019; Tollemar et al., 2018; Vonbrunn et al., 2020). However, there is hardly any literature available on automated image processing to equally facilitate evaluation of mucosal specimen. Especially determining the
expression levels of the major mucosal proteins MUC5AC and MUC5B is imperative to better assess pathologic mucosal conditions, which is of significant interest in both research and diagnostics.

While physiologic amounts of airway mucus are essential to lubricate the respiratory epithelium (REp), increased synthesis and density of airway mucus is a major cause for muco-obstructive pulmonary diseases and develops gradually upon exposure to airborne particles (Fahy & Dickey, 2010; Rubin, 2010; Tian & Wen, 2015). Cigarette smoke is a major source for harmful particles and severely elevates MUC5AC levels in both REp and submucosal glands (SMGs; Goldfarbmuren et al., 2020; Kesimer et al., 2017). However, a physiologic ratio of MUC5AC to MUC5B is a prerequisite for effective respiratory clearance (Hattrup & Gendler, 2008; Rose & Vojnow, 2006; Roy et al., 2014). While MUC5C is produced abundantly in both REp and SMG, MUC5AC is primarily expressed in superficial goblet cells and found in SMG only under pathologic conditions (Okuda et al., 2019; Ostedgaard et al., 2017; Widdicombe & Wine, 2015).

Techniques to analyze mucus composition and properties are rare, require expertise, or special equipment, are often error-prone in their respective collection methods and fail to provide information on spatial expression patterns (Atanasova & Reznikov, 2019). Image-based protocols to visualize and score the overall mucous level in mucus plugging of sputum and evaluate pathologic mucosal areas and assess number, ratio, and distribution of mucus cells, is labor intensive and yields data in an “all-or-nothing” principle rather than intermediate values on staining intensity and ratio of mucosal proteins (Aterman & Norkin, 1963; Gregory et al., 2010; Patel et al., 2018; Sakamoto et al., 2000). Microscopy-based computational evaluation of mucosal specimen could facilitate quantitative analysis while enhancing reproducibility and minimizing inter- and intraobserver variability in interpreting histological images (Osborne et al., 2014; van Diest et al., 1997).

In this study, we present a simple and automated computational approach that is based on standard histological processing and the open-source software Cell Profiler (Carpenter et al., 2006; Kamentsky et al., 2011). In spite of the software’s versatility, it is still often compromised in recognizing integral structures for example, blood vessels or glandular tubes in mucosal specimen. We addressed this issue by adapting established histologic and fluorescent staining techniques, which allowed our developed Cell Profiler pipeline to specifically target individual mucosal areas and assess number, ratio, and distribution of major airway mucins. The pipeline was validated by comparing mucus levels in tracheal tissue and air–liquid interface cell cultures of primary normal human bronchial epithelial cells (NHBE) derived from smoking and nonsmoking individuals.

2 | MATERIALS AND METHODS

2.1 | Specimen collection

Specimen from the distal trachea were collected in accordance with the Austrian law BGBl. 1 Nr. 108/2012 and approved by the ethical committee (Medical University of Graz [MU Graz], EK30-377ex17/18). All samples were obtained from organ donors from the Division of Transplantation Surgery or during autopsies from the Institute of Pathology at the MU Graz. Samples were grouped in either smokers (no medical history in pulmonary diseases, current smoking) or “normal” subjects (neither medical history of pulmonary diseases nor records of smoking). The mean age of donors was 72 ± 9.7 years for the normal (five male and one female) and 63.2 ± 12.5 years for smoking cohort (one male and five female).

2.2 | NHBE isolation and ALI culture

The specimens were washed with 0.9% NaCl/3% penicillin-streptomycin to remove blood and mucus plugs, mechanically separated from soft tissue or lung parenchyma and kept in incubation medium (IM; MEM, 1× MycoZAP, 40 μg/ml tobramycin, 500 μg/ml DTT, 10 μg/ml DNase in PBS) for 4 hr at 4°C before incubation in digestion medium (DM) containing IM supplemented with collagenase solution (185 U/ml collagenase type II, 2 mg/ml BSA, 0.5 mM calcium chloride, 1 U amino acids 100×, 5% FCS in DMEM) for 2 hr at 37°C. The epithelial cells were scrapped off the luminal side of the trachea four to five times into fresh MEM, spun at 200 g for 10 min and incubated in MEM supplemented with 5× Antibiotic-Antimycotic (Gibco) for 2 hr at 37°C. The cells were then resuspended in PneumaCult™ ExPlus medium (Stemcell, Vancouver, Canada) supplemented with 1× MycoZAP (Lonza, Basel, Switzerland) and seeded in gelatine-coated cell culture flasks. The cells were harvested at a confluency of 80–90% and seeded into fibronectin-coated transwell inserts (VWR, Dublin, Ireland), at a density of 3 × 10⁵ cells per 12-well insert, and incubated in MEM supplemented with 5× Antibiotic-Antimycotic (Gibco) for 14 days at 37°C. The medium was changed every other day.

2.3 | Tissue processing

To adequately preserve airway mucus, both tracheal tissue and ALI cultures were fixed using Carnoy’s reagent (ethanol, chloroform, glacial acetic acid; 6:3:1, v/v, [Puchtler, Waldrop, Conner, & Terry, 1968]) or 3.7% paraformaldehyde for 24 hr or 20 min, respectively. Dehydration and tissue processing for embedding in paraffin was conducted automatically using the Excelsior™ AS Tissue Processor (ThermoFisher Scientific). Paraffin-embedded blocks were cut to 5 μm sections using a rotary microtome (HM355 with STS & Cool-Cut, ThermoFisher Scientific, Waltham, MA, USA). The sections were mounted on SuperFrost Plus™ slides, heat-dried at 54°C for 2 hr and 48°C overnight, de waxed using Histobar Clear® (Histoslab®, Askim, Sweden) for four times 5 min each and then rehydrated in a graded series of ethanol (100, 96, 70, and 50%) followed by washing in distilled water three times for 3 min each. Antigen retrieval was performed using 0.1 M
sodium citrate buffer solution (pH 6) for 15 min at 93 °C using a KOS Microwave Multifunctional Tissue Processor (Milestone, Sorisole, Italy).

2.4 | Alcian blue staining and immunofluorescence

For AlB staining, the slides were incubated in 3% acetic acid (v/v) followed by 1% alcian blue (in 3% acetic acid, pH 2.5) for 10 min at room temperature (RT), washed in 3% acetic acid and flushed in water and 1× PBS. No counterstaining was conducted. Directly afterward, immunofluorescence (IF) was performed using the Ultra Vision Protein block (TA-125-UB, ThermoFisher Scientific) for 5 min to reduce background before incubation with primary antibodies against MUC5AC (MA5-12178) and MUC5B (PA5-82342, both 1:100 in antibody diluent, both ThermoFisher Scientific) for 45 min at RT. The selected antibody concentrations for combinational staining of AlB and IF (AlB-IF) were chosen after empirical testing of dilutions reported in literature (1:100, 1:1,000 and 1:10,000; Figure S2, [Burgel, Cardell, Ueki, & Nadel, 2004; Gundavarapu et al., 2013; Kamio et al., 2005; Yan et al., 2008]). Slides were washed three times with 1× PBS and incubated with the secondary antibodies goat-anti-mouse IgG Alexa Fluor 546 (A11003) and goat-anti-rabbit IgG Alexa Fluor 633 (A212070; both 1:400, both ThermoFisher Scientific) for 30 min at RT in the dark. Nuclei were counterstained using 4',6-diamidino-2-phenylindole (DAP, 1:2000, ThermoFisher Scientific). Combined mouse and rabbit IgG (5 μg/ml, diluted in antibody diluent, Agilent Technologies) was used as negative control. The slides were dried using an ascending graded series of ethanol at 70, 90, and 100% for 3 min each and mounted with ProLong™ Gold Antifade Reagent (ThermoFisher Scientific).

2.5 | Image acquisition

Between 170 and 590 image sets covering the entire tracheal area and 20 to 144 image sets covering ALI cultures of each specimen were acquired automatically in five channels each (bright field, DAPI, fluorescein isothiocyanate [FITC], Cy3 and Cy5) within <1 hr (tracheal tissue) and <10 min per ALI culture after defining both area and focal plane using an Observer.Z1 inverted microscope equipped with a Colibri 7 LED illumination system and axiocam cameras for bright field (Axiocam 506) and fluorescence (Axiocam 702 mono) imaging and excitation and emission filter sets for visualization of the aforementioned channels using a 20× objective (LD Plan-Neofluar 20×/0.40 cor., D = 0–1.5 mm) and the ZEN 3 blue software (Version 3.0; all Carl Zeiss, Oberkochen, Germany). Exposure times for bright field and fluorescence imaging were optimized for the smoking cohort using auto-exposure and maintained constant throughout image acquisition across all samples. Brightness and contrast were adjusted for enhanced visualization in representative images only. Original grayscale images were used for downstream analysis using CellProfiler. Image sets that did not cover any mucosal areas, showed inadequate preservation of morphology or contained both SMG and REp in tracheal specimens were excluded to avoid skewing data on spatial expression of mucosal proteins between SMG and REp. The final analysis included 28–98 image sets in SMG, 12–70 image sets in REp and 16–120 image sets in ALI cultures (all 200× magnification). Representative images are shown in both bright field and IF (Figures 1 and 8).

2.6 | Computational analysis of mucosal parameter

Graphical data analysis was performed using the open-source software CellProfiler (version 3.1.5.; [Carpenter et al., 2006; Kamentsky et al., 2011]). A “short” and an “extended” version of the pipeline were developed within this study and uploaded for public access (Figures 2 and 3). The pipelines are designed to first identify the AlB positive area (AlB+) in bright field images to frame the general mucosal area within which the AlB staining intensity as well as the Cy3 (MUC5AC) and Cy5 (MUC5B) fluorescence intensities will be assessed as mean pixel intensities (henceforth referred to as mean intensity). This process calculates the mucin quantity within the total AlB+ area also engrafting regions without mucin content therefore normalizing the mucin signal intensity to the overall mucosal area, which is why combined bright field and IF staining is imperative. The “extended” version of the pipeline complements the “short” pipeline by additionally identifying the area occupied by either MUC5AC (MUC5AC+) or MUC5B (MUC5B+) to return both localization and mean intensity of their local expression level without dilution. Both pipelines are briefly outlined below. Detailed descriptions of both the “short” and “extended” pipeline delineating all parameters are given in the Supporting Information. Apostrophes highlight the modules that build up the pipeline.

2.6.1 | Short pipeline

The original AlB and IF images were loaded into CellProfiler and matched as intended using “NamesAndTypes.” To identify the AlB+ areas as primary objects, the images were split into the individual RGB grayscale channels using the module “ColorToGray” (#1) and processed to distinguish the AlB+ area by subtracting the RGB red (× 1) and RGB green (× 0.1) image from the RGB blue image using the module “ImageMath” (#2). This step generates a negative of RGB red, in which AlB is mainly pronounced while excluding features of the surrounding tissue that are also shown in RGB green. Hence, only areas marked by AlB are emphasized (Figure 2a–e).

The module “IdentifyPrimaryObjects” (#3) subsequently identifies the highlighted area (AlB_calc) using an intensity threshold. Using “OverlayObjects” (#4) allows manually verifying the correct identification of the AlB+ area, which represents the mucosal structures (Figure 2f–h). To accurately measure the fluorescence intensities of MUC5AC and MUC5B within the AlB+ area, the background fluorescence derived from the FITC channel was corrected by using the module “ImageMath” (#5/#7), which subtracts the FITC image from the Cy3 and Cy5 IF images.
and “OverlayObjects” (#6/#8) to monitor the correct positioning of the background-corrected MUC5AC and MUC5B signals (MUC5AC_calc, MUC5B_calc) within the AlB \(^+\) area (Figure 2i–p).

### 2.6.2 Extended pipeline

In case the mucin intensities within the MUC5AC \(^+\) and MUC5B \(^+\) areas are of interest as a measure of local mucin expression, the “extended” version of the pipeline may be used. Here, the mucin signals of the background-corrected IF images (steps #5 and #7 of the “short” pipeline) are identified as primary objects (“IdentifyPrimaryObjects”; #9, #11) returning the MUC5B \(^+\) (Figure 3a–c) or MUC5AC \(^+\) (Figure 3e–g) areas, which may be manually verified by “OverlayObjects” (#10, #12, Figure 3d,h). Finally, the module “MeasureObjectIntensity” (#9/#13) allows calculating the area and staining intensities while “SaveImages” (#10/#14) and “ExportToSpreadsheet” (#11/#15) are added to export the resulting images and data. These three modules may be modified as required. The pipeline listed as modules in Cell Profiler is shown in Figure S3.
2.7 Validation of pipelines

The pipelines were validated manually for the accurate and complete identification of the individual mucosal areas by classifying the detected primary objects either as “correctly detected” (complete detection of AlB+ area), “partly detected” (incomplete detection of AlB+ area), “false positive” (excessive area detected outside AlB+ area) or “false negative” (AlB+ area not detected).
The ratio is given in percent of mucosal structures present within each individual image set \( N = 12 \) including areas counted as false positive \((\pm 100\%)\). The AlB\(^+\) area in ALI cultures was correctly detected in 100\% of images and was therefore not further evaluated.

### 2.8 Data analysis and statistics

GraphPad Prism version 8.0.2 (GraphPad Software, http://www.graphpad.com) was used for statistical analysis and data representation. Data are shown as means ± standard deviation (SD). Data were tested for normality using Kolmogorov–Smirnov test followed by two-tailed Student’s test for normally distributed data or one-way ANOVA with Tukey post-hoc test if >2 groups were compared. Kruskal–Wallis with Dunn’s multiple comparison testing was used for data not following Gaussian distribution and small sample sizes \( N < 6 \). Correlation analysis was conducted using nonparametric Spearman \( r \) test. Significance levels were considered at \( p < .05 \) (*), \( p < .01 \) (**), \( p < .001 \) (***) and \( p < .0001 \) (****).

### 3 RESULTS AND DISCUSSION

In order to visualize and evaluate the complete mucosal area while detecting the individual mucosal proteins MUC5AC and MUC5B directly on the same slide, we combined general staining methods on mucus for bright field imaging with targeted staining of mucosal proteins in IF. Downstream computational analysis of such color-coded individual targets allows direct comparability of staining intensities and fractions of airway mucins within the mucosal area. The entire workflow and timeline is schemed in Figure 1a.

### 3.1 Sample preparation and staining conditions

Unmitigated fixation of airway mucus is a prerequisite for downstream mucosal analysis. While 3.7\% paraformaldehyde did not properly retain airway mucus especially on ALI cell cultures (Figure S1), we found the mucus integrity well preserved in Carnoy-fixed samples and proceeded using only the latter. We initially attempted to combine a general mucosal staining such as PAS or AlB with IF (PAS–AlB–IF) and
therefore tested the specificity of the primary antibodies for MUC5AC and MUC5B in immunohistochemistry in comparison to PAS–AlB–IF.

MUC5AC staining in normal IHC was highly specific at several commonly used concentrations (1:100–1:1,000, [Burgel et al., 2004; Gundavarapu et al., 2013; Kamio et al., 2005; Yan et al., 2008]) and was even detected at a dilution of 1:10,000 (Figure S2a).

However, PAS–AlB–IF did not return any staining of mucosal proteins (data not shown). This is most likely due to alterations of the epitopes during the oxidation reaction by periodic acid, which might hinder antibody binding. In contrast, linking AlB staining with IF yielded proper staining of MUC5AC and MUC5B at primary antibody dilutions of 1:100 (final concentrations 2 μg/ml for MUC5AC and 1 μg/ml for MUC5B, Figure S2b), which were therefore chosen for combinational staining and downstream analysis. Representative images of tracheal tissue of bright field and IF staining from the same slide are shown in Figure 1b (nonsmoker) and Figure 1c (smoker).

Perhaps the different antibody binding capacity following PAS or AlB staining is due to the lesser degree of epitope modification under applied conditions (concentration, incubation time, acidity) by acetic acid (AlB staining; 3%, 10 min, \(pK_a=4.75\)), than by periodic acid (PAS reaction; 2%, 60 min, \(pK_a=1.64\)). The exact causality of the dissimilar antibody binding affinity, however, remains to be determined empirically.

### 3.2 Detection of mucosal areas

**CellProfiler** pipelines were developed to allow discerning the target areas based on their color and staining intensity using the same manually optimized global threshold across various samples (Figures 2 and 3). It was therefore necessary to validate the process of identifying mucosal structures in both effectiveness and accuracy. Our **CellProfiler** pipelines detected the mucosal area based on AlB staining with an overall precision of 83% for correctly or partly detected SMG and in 81% of REp (Figure 4a,b). Since the areas that contributed to false results were generally small or showed a low level of staining intensity potentially caused by peripherally cut SMG, downstream analysis would only be affected to a minor degree. Identifying AlB+ areas in ALI cultures was accurate across the image set and thus not further validated.

### 3.3 AlB+ area and mean intensity in SMG and REp

The verified pipelines were applied to the image data sets derived from nonsmoking and smoking individuals (Figure 2). Both the AlB+ area and the AlB mean intensity within the SMG and REp were similar between nonsmokers and smokers \((N = 6\) per group; \(N = 5\) for REp in the normal group, Table 1), allowing direct comparison of downstream calculations on mucin content within AlB+ areas. As expected, the AlB+ areas were generally larger in SMG than in the REp, while the REp showed stronger AlB mean intensities (Table 1, Figure S4). These results nicely reflect the correlation between the optical densities of the basic AlB dye that binds to acidic glycosaminoglycans in the form of muco-polysaccharides in these different locations. Mucus is condensed before secretion leading to more intense stains in goblet cells primarily present in the REp (Fahy & Dickey, 2010).

### 3.4 MUC5AC levels in SMG multiply 18-fold in smokers

MUC5AC expression levels of smokers were significantly increased within AlB+ areas in the SMG compared to nonsmoking individuals (Figure 5a), which was not the case in the REp (Figure 5b). Similarly, overall MUC5B levels surpassed MUC5AC in the SMG but not REp, impartial of smoking (Figure 5a,b).

Our findings correspond to an overall 18-fold increase of MUC5AC in the SMG of smokers compared to nonsmokers (Figure 5c). No significant effect was observed for MUC5AC in the REp nor in MUC5B levels in either SMG or REp (Figure 5c,d). Therefore, we were not only able to confirm the elevated mucin levels in the SMG of smokers to be predominantly comprised by MUC5AC as frequently reported (Kanai et al., 2015; Li & Ye, 2020; Rose &

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**FIGURE 4** Validation of identifying the mucosal area based on AlB staining using **CellProfiler**. Overall, 83% in the SMG (a) and 81% in REp (b) were found to be either correctly or partly detected. (c) A representative image of the validation process is shown.
Voynow, 2006), but quantify these elevated levels to be in the range detected in the literature using other methodologies (between 2.3-fold and 10-fold; Kesimer et al., 2017; Wang et al., 2012).

In contrast to other studies, we found only a minor effect on MUC5B levels. It is reported in literature that both reduced (Goldfarbmuren et al., 2020) and elevated levels (Kesimer et al., 2017) were found to be a consequence of cigarette smoking. A reason for this variation may be the differing methodologies and location, as these studies measured MUC5B levels using transcriptomics in the tracheal epithelium and mass spectrometry of sputum, respectively, rendering direct comparison difficult.

Since the pipeline enabled the quantification of both mucins from within exactly the same area, we could use this data to directly assess their ratio and calculated a significant 16-fold increase of MUC5AC to MUC5B levels within the SMG of smokers (Figure 5e). Our study indicated MUC5B levels to remain rather stable upon exposure to cigarette smoke, which suggests that the increased ratio is almost entirely attributed to elevated MUC5AC expression. Increased levels of MUC5AC and MUC5AC/MUC5B ratio have also been reported in other mucosal pathologies such as asthma, opening the proposed methodology to its application in a wide range of respiratory diseases beyond smoking (Jackson et al., 2020).

### TABLE 1

| Group   | SMG (μm²) Mean ± SD | REp (μm²) Mean ± SD | SMG MUC5B Mean ± SD | REp MUC5B Mean ± SD |
|---------|---------------------|---------------------|---------------------|---------------------|
| Normal | 10,175 ± 3,438       | 4,532 ± 2,859       | 0.227 ± 0.096       | 0.334 ± 0.0877     |
| Smoker | 9,847 ± 3,018        | 4,075 ± 6,353       | 0.1869 ± 0.0741     | 0.3127 ± 0.0717    |

Significance levels are stated in italic and marked as bold if statistically significant at \( p < .01 \) (**).
3.5 | MUC5AC elevation increases mucin area rather than density

In addition, the “extended” pipeline allowed assessing whether the increased MUC5AC levels in the SMG of smokers attributes to either the expansion of MUC5AC + areas or the local increase in MUC5AC signal intensity by interrogating the spatial distribution and the staining intensity of the confined area that the mucosal proteins actually inhabits.

MUC5B was abundantly present and homogeneously distributed throughout the SMG and REp image sets as well as across all investigated patient cohorts (Table 2). MUC5AC, on the contrary, showed strong heterogeneous distribution across images, the area it occupies within the SMG, and between smokers and nonsmokers (Table 2). Precisely, we found MUC5AC in the SMG in 55% of images from smokers compared to 14% in three out of six normal individuals that occupied 8% and <1% of the AlB + area, respectively. No difference was seen in the REp image sets (Table 2).

Further, we used the “extended” pipeline to test whether the staining intensity of the mucins within the respective mucin + areas increase locally upon elevated expression. We found that the localized signal intensity for both mucins was rather stable in smokers compared to nonsmokers in both the SMG and REp (Figure 5c,d). Together, these results suggest that increased MUC5AC levels in smokers (Figures 1b and 5a) primarily expand the area occupied by the mucin rather than thickening its local density.

3.6 | Partial MUC5AC elevation in normal donors

As mentioned before, we identified MUC5AC in the SMG in three out of six normal donors. This was surprising since the general understanding of previous studies is that MUC5AC expression in the SMG occurs only in smokers and does not take place there for normal nonsmokers (Okuda et al., 2019; Widdicombe & Wine, 2015). However, the detected MUC5AC + areas were clearly distinguished from the background and targeted toward AlB + cells in the SMG (Figure 6a). We therefore rated these signals as MUC5AC specific and sub-grouped the donors as either normal5AC - or normal5AC + for downstream analysis.

While MUC5AC levels per AlB + area were significantly distinct comparing normal5AC - and normal5AC + donors to smokers individually, we found no statistic difference when comparing normal5AC - to normal5AC + specimen, warranting to merge the subgroups for analysis (Figure 6b,c).

Nevertheless, our results prompted an intense literature search, which indeed revealed a certain inconsistency regarding MUC5AC in the SMG of normal individuals because some studies also reported sparse amounts of MUC5AC in the SMG of healthy donors to occupy about 2% of the total mucosal area (Caramori et al., 2009; Inoue et al., 2008). This percentage is even higher than the <1% observed here.

Although these differences might be attributed to the genetic make-up of donors or the used methodology during analysis, our findings reflect the inhomogeneous distribution of MUC5AC in SMG as it was found in only 14% of total images in only three out of six donors and might have been missed if only single images were analyzed. We therefore strongly recommend comprehensive analysis comprising wide areas of the specimen over single images, as done in this study. Additionally, these variations highlight the sensitivity of the pipeline as its threshold was optimized to also detect weak signals of MUC5AC.

3.7 | Positive correlation of MUC5B and MUC5AC expression in smokers and nonsmokers

Since the expression of both MUC5AC and MUC5B is reported to be induced by cigarette smoke (Goldfarbmuren et al., 2020; Kesimer...

| Group | Mucin + images in % of images per data set | Mucin + area within mucin + images (in % of AlB + area) |
|-------|------------------------------------------|-------------------------------------------------|
|       | SMG | REp | SMG                       | REp                       |
| Normal | 100% | 100% | 89.24 ± 28.38 | 163.70 ± 28.98 |
| Smoker | 104.10 ± 20.36 | 268.40 ± 173.50 | p = .3231 | p = .4682 |
| p-value | / | / | p = .0049/** |
| MUC5AC | SMG | REp | SMG                       | REp                       |
| Normal (n = 3/6) | 14.11 ± 1.12 | 94.29 ± 7.04 | 0.50 ± 0.16 | 212.8 ± 246.1 |
| Smoker | 55.37 ± 25.32 | 100% | 7.96 ± 11.88 | 134.6 ± 87.13 |
| p-value | p = .0103/* | p = .1074 | p = .1847 | p = .4828 |
| p < .0001/**** |

Significance levels are stated in italic and marked as bold if statistically significant at p < .05 (*), p < .01 (**), p < .001 (***), and p < .0001 (****).
et al., 2017; Kim et al., 2015), we tested for a potential associated expression. Spearman rank correlation revealed a moderate positive correlation of MUC5AC and MUC5B expression in both smokers \( r = .6571 \) and nonsmokers \( r = .5429 \); Figure 7), which confirmed previous studies.

### 3.8 Pipeline confirms REp as preferential expression site of MUC5AC

Previous studies report the REp to be the preferential expression site for MUC5AC (Caramori et al., 2009; Inoue et al., 2008; Okuda et al., 2019; Ostedgaard et al., 2017; Widdicombe & Wine, 2015). Image analysis with the proposed pipeline confirmed these findings reporting both the percentage of images with MUC5AC+ areas and the percentage of MUC5AC+ area within the total AIB+ area to be significantly increased in the REp (Table 2). Interestingly though, the MUC5AC+ area in the REp was larger than the AIB+ area potentially indicating MUC5AC distribution throughout areas not detected by AIB staining. This may be either because AIB staining targets acidic polysaccharides leaving areas with neutral polysaccharides partly undetected, or an inappropriate threshold, as the pipeline was optimized to detect the SMG area and maintained for the REp data sets to allow comparison. Hence, this limitation could be minimized by optimizing the threshold for the REp data set, but should be kept in mind when using the proposed pipeline.
3.9 Automated mucus analysis in 3D ALI cell cultures

While the determination of mucosal compositions in tracheal tissue is of great value in digital pathology and retrospective analysis of clinical study cohorts, tissue samples themselves are severely limited when used to develop and test new therapeutics. To fill that gap, controllable 3D organoid models such as the ALI cell culture system are increasingly applied in studies concerning host-pathogen interactions (Caves et al., 2018; Marrazzo et al., 2016), drug efficiency (Min et al., 2013) or mucus production (Castellani, Di Gioia, Di Toma, & Conese, 2018) under various conditions (Miller & Spence, 2017), while minimizing the use of animal models in basic research (Derakhshifard et al., 2018; Pfeiffer et al., 2014; Sosa-Hernández et al., 2018).

In order to demonstrate the broad applicability of our developed method, we applied the proposed pipelines to reconstituted ALI cell cultures of primary NHBE derived from both smokers and non-smokers to evaluate potential mucus-modulating differences resulting from exposure to cigarette smoke prior to isolation and ALI establishment (Figure 8a,b).

We found no difference in the AlB⁺ area when normalized to the membrane length but a trend toward increased AlB intensity in ALIs derived from smokers. The ratio of MUC5AC to MUC5B was not significantly different between smokers and non-smokers. The fold change of mucins in ALI cultures derived from smokers was not significantly different from those derived from non-smokers, as shown in Figure 8d and 8e.

**FIGURE 8** Automated mucus analysis of air-liquid interface (ALI) cultures using the proposed method. (a) Representative AlB and IF images of the ALI culture with staining of MUC5B (yellow), MUC5AC (red), and nuclei (blue). (b) Selected steps of the “short” (Figure 2) and “extended” (Figure 3) pipeline illustrating off-set calculations (1–3) and detection (4–6) of the mucosal area as well as background correction and detection of MUC5AC (7–10), representative for both mucins. (c) Raw data of detected mucin levels per AlB⁺ area and mucin⁺ area comparing ALI cultures derived from either smoker (n = 3) or nonsmoker (n = 3). (d) Fold change and (e) ratio calculation of MUC5AC and MUC5B per AlB⁺ area. Significance levels were considered at p < .05 (*)
TABLE 3  AlB\(^+\) area and mean intensity as well as mucin\(^+\) area in % of AlB\(^+\) area of ALI cultures derived from smokers and nonsmokers

| Group  | Mucin\(^+\) area in % of AlB\(^+\) area | MUC5AC | MUC5B |
|--------|---------------------------------------|--------|--------|
| Normal | Smoker                                |        |        |
|        | Normal                               | 101.6 ± 30.09 | 17.76 ± 13.82 |
|        | Smoker                               | 44.73 ± 30.73 | 17.76 ± 13.82 |
|        | p = 0.2027                           | p = 0.3841 |        |

Note: Values are given as means ± SD. Significance levels are stated in italic and were considered as ‘trend’ at \(p<0.08\)(#).

3.10  General discussion

Overall, our developed methodology proved generally robust as both pipelines returned comparable values on target identification and staining intensities across different specimen of tracheal tissue and ALI cell cultures and allowed to quantify major mucosal parameters using only standard histological staining.

Although the data are derived from 2D images and cannot be related directly to the actual fraction of mucosal solids as derived by bronchoscopy or bronchoalveolar lavage and western blotting (Atanasova & Reznikov, 2019), the fold change on increased MUC5AC levels found in our study was very similar to those previously reported.

Still, the pipeline shows difficulties for example in distinguishing granular and secreted mucus when analyzing surface epithelial structures or ALI cell cultures. Secretion causes hydration and hence swelling of airway mucus, which in turn, enlarges the AlB\(^+\) area while reducing the staining intensity (Fahy & Dickey, 2010). Consequently, the overall values on mucosal protein levels in the REp and ALI cultures is lower when the entire AlB\(^+\) area is analyzed. This limitation may be addressed by adjusting the threshold of mucus detection or generating image masks that accurately define the target area.

Abnormalities in mucus quantity and protein content are not only a phenomenon of lung pathologies, but also common in the gastrointestinal and the reproductive tract (Davis, 2006; Rowe, Miller, & Sorscher, 2005; Viniol & Vogelmeier, 2018). Because mucopolysaccharides are also contained within mucus of these tissues, it might be possible to target them with the proposed pipeline. Furthermore, the primary antibodies used within the proposed method are interchangeable, widening the scope of experimental settings or therapeutic interventions when used on in vitro models such as ALI cell cultures (Castellani et al., 2018; Upadhyay & Palmbreg, 2018) or animal models (Mercel et al., 2020). We commend the reported pipelines as a valuable and promising tool for quantitative image-based analysis to be used to facilitate research on mucob-structive diseases.

4  CONCLUSION

We developed Cell Profiler pipelines that enabled the automatic quantification of airway mucus including expression levels of the major mucosal proteins MUC5AC and MUC5B by using standard histological techniques. In this way, the ratio of the mucosal proteins within the mucosal areas of the SMG, REp and reconstituted ALI cell cultures could be calculated. By comparing the mucosal characteristics of nonsmokers and smokers, our method confirms the results of previous studies reporting a significant increase in MUC5AC levels upon exposure to cigarette smoke and among the first times, add numerical values to image-based mucus analysis: smokers possessed 18-fold higher levels of MUC5AC expression resulting in an overall 16-fold increase in the MUC5AC to MUC5B ratio. Notably, MUC5B levels surpassed MUC5AC in both SMG and ALI cultures independently of smoking.

Therefore, the proposed pipelines provide an innovative, robust, and high-processing computational tool to quantify so far only histologically visualizable objects. Since these pipelines are freely available online and user-friendly, they expand the computational toolbox on image-based analysis in research fields focused on mucob-structive conditions.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.
AUTHOR CONTRIBUTIONS
Silvia Groiss and Dagmar Brislunger designed the experiments. Philipp Stiegler, Bettina Leber, and Bernadette Liegl-Atzwanger collected patients’ material. Silvia Groiss, Ina Somvilla, Julia Fuchs, and Christine Daxböck processed patients’ material and performed the experiments. Silvia Groiss, Ingrid Lang-Olip, and Dagmar Brislunger analyzed the data and wrote the manuscript. Silvia Groiss designed the graphics. All authors critically reviewed the manuscript.

DATA AVAILABILITY STATEMENT
The data that supports the findings of this study are available from the corresponding author on reasonable request. The pipelines and template data sets are freely available at https://cellprofiler.org/examples/published_pipelines.

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Supporting Information

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