Title: Modified De-Epithelialization Protocol Enhances Short-Term Chondrocyte Survival in Chimeric Tracheal Allografts

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1. We propose a new de-epithelialization protocol that adequately removes epithelial, mucosal, and submucosal cells while maintaining a greater proportion of viable chondrocytes.
2. The new protocol showed a significant ($p < 0.05$) increase in chondrocyte viability up to four days after de-ep when compared to the original protocol
3. While not without limitations, our new protocol may be used to engineer chimeric tracheal allografts without the need for cartilage regeneration.

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ABSTRACT.

Background: Tracheal transplantation is indicated in cases where injury exceeds 50% of the organ in adults and 30% in children. However, transplantation is not yet considered a viable treatment option partly due to high mortality and morbidity associated with graft rejection. Recently, decellularization (decell) has been explored as a technique for creating bioengineered tracheal grafts. However, risk of post-operative stenosis increases due to the death of chondrocytes, which are critical to maintain the biochemical and mechanical integrity of tracheal cartilage. In this project, we propose a new de-epithelialization protocol that adequately removes epithelial, mucosal, and submucosal cells while maintaining a greater proportion of viable chondrocytes.

Methods: The trachea of adult male outbred Yorkshire pigs were extracted, decontaminated, and decellularized according to the original and new protocols before incubation at 37 °C in DMEM for 10 days. Chondrocyte viability was quantified immediately post-decellularization and on days 1, 4, 7, and 10. Histology was performed pre-decellularization, post-decellularization, and post-incubation.

Results: The new protocol showed a significant ($p < 0.05$) increase in chondrocyte viability up to four days after de-ep when compared to the original protocol. We also found that the new protocol preserves ECM composition to a similar degree as the original protocol. When scaffolds created using the new protocol were re-epithelialized, cell growth curves were near identical to published data from the original protocol.

Conclusion: While not without limitations, our new protocol may be used to engineer chimeric tracheal allografts without the need for cartilage regeneration.

Key Words: tissue engineering, decellularization, allograft, trachea, bioreactor, regenerative medicine, chondrocyte, stem cell, graft, transplantation, transplant, surgery, bioengineering, stenosis, cartilage, viability, cell viability (Source: MeSH-NLM).

INTRODUCTION.

Tracheal transplantation is indicated in cases where injury exceeds 50% of the organ in adults and 30% in children.[1] However, tracheal replacement therapy is currently considered a high-risk procedure, mostly offered as a treatment option on compassionate use cases. A major reason behind the relatively high rate of complications is the plethora of immunological compatibility issues created by orthotopically transplanting a donor organ. A possible solution to this problem may be found in tissue engineering-based approaches for whole-trachea regeneration. Recently, significant progress has been made in engineering bioartificial organs *de novo* from pluripotent stem cells and acellular extracellular matrix (ECM) scaffolds.[2–5] Somatic cells have been differentiated into functional lung epithelial cells after transformation into induced pluripotency.[6] Also, stem cell-seeded tracheal grafts from cadaveric donors have been transplanted into patients with end-stage airway diseases.[3] Despite these milestones, recellularized tracheal allografts still demonstrate increased risk of stenosis, resulting in post-operative complications.[2,3,7]

Decellularization (decell) of donor trachea is a relatively well-studied technique for creating natural scaffolds for whole-trachea regeneration.[8–14] One such decell approach involves the use of detergents to remove donor cells from a cadaveric trachea, leaving behind the ECM scaffold.[10,15,16] Recipient-derived induced pluripotent stem cells (iPSCs) may then be seeded onto such scaffold, reconstituting the respiratory epithelium.[3] The benefits of this approach are twofold. Firstly, risk of graft rejection is reduced because the immunogenic donor tracheal epithelium and submucosa are removed and replaced with autologous cells.[7,9,17–19] Secondly, the use of a native biological scaffold rather than synthetic materials preserves the important tissue architecture and ultrastructure, which allows us to better mimic the cellular niche later during scaffold seeding.[16] However, the full thickness decell protocols currently used are harmful to chondrocytes, leading to deficiencies in the biochemical and mechanical integrity of hyaline cartilage.[13,16,20] This may increase risk of post-operative stenosis and other complications upon implantation.[21] To address this issue, the Waddell lab uses a de-epithelialization (de-ep) technique pioneered by Aoki *et al.* to remove only the immunogenic epithelium while maintaining chondrocyte viability.[16,22] This de-ep can be followed by re-ep using autologous cells to produce chimeric tracheal allografts.

Despite these advances, the original de-ep protocol is suboptimal because it results in a relatively low chondrocyte survival (68.6 ± 7.3%).[16] A new de-ep protocol has recently been developed based on the postulated chemical and osmotic effects of various decellularization fluids on chondrocytes. This protocol is believed to provide milder de-ep conditions that may increase chondrocyte survival while providing similar removal of epithelial cells. When designing this new protocol, we made the following hypotheses: 1) Removal of the standard 40 min ddH_2O wash cycle will decrease osmotic stress on SDS-perforated cells, and 2) Using decreasing concentrations of SDS rather than a static concentration will remove greater amounts of residual SDS in submucosal tissue, protecting cartilage. An initial high concentration (1%) is required for decellularizing epithelium and mucosa, after which lower concentrations (0.1%, 0.01%) are more appropriate for minimizing damage to cartilage. This study intends to serve as a proof-of-concept to demonstrate that a modified de-ep protocol can allow for the removal of immunogenic tissue (epithelium, mucosa, submucosa, and perichondrium) while preserving more of the chondrocyte population. The objectives of this study are to: 1) evaluate chondrocyte viability in porcine trachea after the new de-ep protocol, 2) validate the new protocol's ability to preserve ECM biochemical composition, and 3) validate the new protocol's ability to support epithelial cell attachment and viability during re-ep. We hypothesize that the new protocol will produce de-epithelialized

scaffolds with improved chondrocyte viability while demonstrating similar biochemical composition and re-epithelialization performance compared to the current protocol.

METHODS.

Tracheal extraction

Adult male outbred Yorkshire pigs (30-40 kg) (n = 6) were used as donor animals due to the physiological similarity of their cardiopulmonary system to that of humans. After anesthesia by isoflurane administration, a median incision of the neck was made to expose the larynx and upper trachea. Next, a median sternotomy was performed to open the chest wall and provide access to the lower trachea. Using Mayo scissors, the trachea was bisected just below the cricothyroid membrane and lifted away from the esophagus. Surrounding connective tissue was dissected away using curved Mayo scissors. To detach the trachea, the left and right main bronchus were bisected just below the carina. The extracted trachea was immediately placed in decontamination solution at 0 °C until transported out of the operating room. The decontamination solution contained Hank's balanced salt solution (HBSS) supplemented with 2% (w/v) bovine serum albumin (BSA), fluconazole (4 µg/mL), colistimethate (5 µg/mL), imipenem/cilastatin (25 µg/mL), ceftazidime (154 µg/mL), penicillin (200 U/mL), streptomycin (200 µg/mL), amphotericin B (2.5 µg/mL) and gentamicin (50 µg/mL). The tracheas were subsequently incubated at room temperature on a rocking platform (30rpm) for 2 hrs. After this incubation, the decontamination solution was replaced with fresh solution, and luminal mucus was scraped off using a micro-tapered stainless-steel spatula. The tracheas were incubated at 4 °C overnight until de-ep was performed the next morning.

All animals received humane care in compliance with the ''Principles of Laboratory Animal Care'' formulated by the National Society for Medical Research and the ''Guide for the Care of Laboratory Animals'' published by the National Institutes of Health. The study was approved by the Animal Care Committee of the Toronto General Research Institute.

De-epithelialization and incubation

The following solutions were prepared under sterile conditions and adjusted to a pH of 7.4: 1%, 0.1%, and 0.01% sodium dodecyl sulfate (SDS); 1% triton X-100; Dulbecco's phosphate buffered saline (DPBS). A perfusion system was constructed using PVC tubing and 4-way Luer connection stopcocks as illustrated in **figure 1** for the original de-ep protocol and **figure 2** for the new de-ep protocol. A rotating perfusion bioreactor was used, modified from Haykal *et al.* Using three 2/0 silk sutures, the trachea was anastomosed to the bioreactor with its proximal end facing the inlet of the chamber **(figure 3)**. De-ep was performed according to the original, new, and control protocols outlined in **tables 1-3**. Following de-ep, the proximal and distal ends of the trachea were trimmed such that only the portions exposed to the decellularization media were used for the 10-day subsequent incubation. The tracheal segments were then placed in decontamination solution for 48 hrs at 4 °C on a rocking platform (30 rpm). Finally, the tracheae were incubated at 47 °C with 5% CO_2 in a 250 mL Erlenmeyer flask fitted with a 20-micron filter allowing for gas exchange. The media used was Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), fluconazole (4 µg/mL), colistimethate (5 µg/mL), imipenem/cilastatin (25 µg/mL), ceftazidime (154 µg/mL), penicillin (200 µg/mL), streptomycin (200 µg/mL), amphotericin B (2.5 µg/mL) and gentamicin (50 µg/mL). Media was changed every 48 hr.

To accurately compare the two de-ep protocols being tested, two control groups were employed. The first control was a decontaminated native trachea that immediately underwent static incubation for ten days without any de-ep procedure. The second control was exposed to the same conditions as the trachea that underwent the new protocol, except with DPBS replacing all steps that required SDS **(table 3)**. Three biological replicates (n = 3) each were performed for the original protocol, the new protocol, and the two control groups.

Histological analysis

Histological samples were taken from the trachea before de-ep; after de-ep; and after incubation **(figure 4)**. Specimens were fixed with 4% paraformaldehyde for 24 hrs and processed with an automated vacuum tissue processor (Leica). Tissue was sectioned into 5 µm slices and stained with hematoxylin and eosin (H&E), Masson's trichrome, Verhoeff's elastin, and Alcian blue.

Chondrocyte viability staining

Chondrocyte viability was quantified immediately after de-ep and on days 1, 4, 7, and 10 **(figure 4)** using a live/dead assay according to manufacturer instructions (ThermoFisher).

Quantification of chondrocyte viability

2-3 rings were obtained from each trachea for a membrane integrity-based viability assay. The mucosa and submucosa were dissected away from the cartilage using fine forceps. The cartilage ring was opened and manually cut in cross section into thin (<1 mm) slices. An ethidium homodimer assay (ThermoFisher) was performed as per manufacturer directions. The slices were imaged under confocal microscopy at 20x magnification. Images were then examined manually by a blinded experimenter. Portions of the image containing viable chondrocytes were circumscribed and the area calculated. The percentage viability of an image was calculated through the following formula:

$$\%\ chondrocyte\ viability = \frac{Area\ of\ viable\ chondrocytes}{Total\ cartilage\ area} \times 100\%$$

Three technical replicates were performed per trachea.

Re-epithelialization

The de-ep bioreactor circuitry from Haykal *et al.* was modified to include media reservoirs for oxygenation, in addition to syringe ports for media changes and sample collection. A 1 mL suspension of BEAS-2B human bronchial epithelial cells (~1×10^6 cells/cm^2) was injected into the lumen. Cells were allowed to adhere for 2 hrs under bidirectional flow at a rate of 1.5 mL/min. After the initial 2 hrs, we started unidirectional perfusion of the lumen at the same rate for seven days. During re-ep, media in the luminal circuit (30 mL) was changed every 24 hrs and media in the outer circuit (250 mL) was changed every 48 hrs.

Cell proliferation activity assay

Cell proliferation during re-ep was measured using a resazurin-based cell viability assay as per manufacturer instructions (PrestoBlue®, Invitrogen). Briefly, a 20 mL solution of 1:20 (v/v) PrestoBlue/DMEM + 10% FBS was prepared. Three 0.5 mL volumes were separated for use as a negative control. The remaining 18.5 mL of reagent was injected into the luminal perfusion circuit of the bioreactor and allowed to circulate for 1

hr. Afterwards, the PrestoBlue solution was aspirated out of the luminal circuit and aliquoted into three 0.5 mL replicates in a 24-well plate for fluorescence analysis at 560 nm (Cytation™ 5, BioTek Instruments).

Statistical analysis

A 2-way analysis of variance (ANOVA) was used to determine statistically significant data ($p \leq 0.05$), with Tukey's *post hoc* multiple comparisons test. Values in figures are presented as means with standard deviations (SD).

RESULTS.

Quantification of chondrocyte viability

There exists an overall negative correlation between days since de-ep and percentage chondrocyte viability **(figure 5)**. Both the original and new protocols significantly reduce viability compared to the two negative controls. However, the new protocol provides significantly improved viability compared to the original protocol in the first four days, after which there is no detectable difference. The most marked improvement in chondrocyte viability occurs on day 4 (61.3±10.8% vs 40.7±5.7%), yet the benefit of the new protocol towards chondrocytes is seen as early as immediately after de-ep on day -2 (78.1±4.7% vs 61.5±10.7%). In other words, long-term chondrocyte survival remains unchanged. Qualitative inspection of live/dead staining reveals the most chondrocyte death at the luminal surface of each cartilage ring **(figure 6)**. There appears to be a smaller "wavefront" of chondrocyte death in the new protocol compared to the current protocol. The average chondrocyte viability of two replicates (n=2) after a 7-day re-ep was 63%.

Histological analysis

In the native trachea control, H&E staining showed the expected pseudostratified columnar epithelium with cilia and goblet cells **(figure 7)**. In both the original and new de-ep protocols, H&E showed a denuded epithelium, with no residual cellular material. No nuclei or cytosolic elements were found in the epithelium. However, both protocols resulted in some nuclei remaining in the deep submucosal regions. Residual acinar gland cells were also visible in both protocols. The hyaline cartilage appears morphologically unchanged.

Masson's trichrome stain showed good collagen preservation throughout the ECM in both the original and new protocols **(figure 8)**. Keratin fibers in the deep submucosa appear better preserved in the new protocol. Verhoeff's elastin stain showed good preservation of elastin fibers in the mucosa and submucosa of both the original and new de-ep protocols **(figure 9)**. Alcian blue stain showed good preservation of acidic polysaccharides such as glycosaminoglycans in cartilage, in both the original and new protocols **(figure 10)**.

Cell proliferation activity assay

When the new protocol's re-ep cell proliferation curve is compared with that of the original protocol from Aoki *et al.*, there is similarity in the rate that fluorescence increases **(figure 11)**. The difference between the two growth curves is nonsignificant as indicated by a multiple t test (false discovery rate approach). Although not a focus of this study, chondrocyte viability after the 7-day re-ep with BEAS-2B was evaluated with two tracheae (n=2). The average chondrocyte viability was 63%.

DISCUSSION.

It has been demonstrated in previous literature that sodium dodecyl sulfate (SDS) reduces cell viability by acting as an anionic detergent, perforating the cell membrane and causing osmotic lysis.[10,11,21] The original protocol contains a 3 hr 1% sodium dodecyl sulfate (SDS) wash that can leave residual detergent trapped in tissue, thus causing ongoing damage after the protocol is terminated. Furthermore, the original protocol includes a 30 min ddH_2O wash that can cause further chondrocyte death via osmotic imbalance leading to cytolysis. Our new protocol made two changes to the original protocol: 1) the 3 hr SDS cycle has been replaced with three 1 hr cycles at decreasing SDS concentrations (1%, 0.1%, 0.01%), and 2) The 30 min ddH_2O wash has been removed. It is believed that the first change limits deep penetration of residual SDS into tissue, while the second change reduces cytolysis of chondrocytes. In other words, this new protocol was designed to provide milder de-ep conditions that increase chondrocyte survival while providing similar removal of epithelial cells. Both negative controls (native trachea and DPBS-only de-ep trachea) showed close to 90% viability. Therefore, it seems that SDS retention in the ECM is a major contributor to chondrocyte death after de-ep, overshadowing the cytolytic effect of the ddH_2O wash and other potential minor contributors. Attempts at quantifying the amount of residual SDS in de-epithelialized tissues using a methylene blue assay were unsuccessful. Future studies should investigate the relationship between residual SDS levels and chondrocyte viability. The short-term nature of the improvement in chondrocyte viability observed in this study was likely due to an initial reduction in residual SDS concentration in submucosal tissues, followed by eventual permeation of the SDS through submucosa and into cartilage due to passive diffusion. Confocal images of the cell viability assay show a clear delineation between calcein-AM (live) cells and ethidium homodimer-1 (dead cells), suggesting a progressive “wavefront” of cell death that is consistent with diffusion of residual SDS. Confirmation of this theory is required, although preventing the diffusion of SDS through submucosal tissue would be difficult or impractical to accomplish in any de-ep protocol.

Examination of H&E slides shows that both protocols were extremely efficient at denuding the epithelium. However, neither protocol appears to sufficiently decellularize acinar glands. Furthermore, the new protocol seems to be less efficient at decellularizing deep submucosal layers. This result was expected since our new protocol uses decreasing concentrations of SDS and is less aggressive overall compared to the original protocol, among others.[23,24] Therefore, with the current detergent-based methods of de-ep, the goal of selectively preserving chondrocyte viability seems to depend on the careful titration of SDS concentrations, walking a fine balance between over- and under-decellularization. Our study shows that the new protocol sacrifices decellularization performance in return for better chondrocyte survival.

Previous studies have shown that decellularization cycles can reduce several ECM components that are critical to structural integrity, including elastin, collagen, and glycosaminoglycans.[16,24,25] Qualitative histological analysis demonstrated that our new protocol is not any more damaging to ECM components than the original protocol. Elastin, collagen, and glycosaminoglycans were found to be preserved after de-ep to a similar degree as with the original protocol. Tracheal compliance and viscoelasticity were not tested because previous studies by Aoki *et al.* have confirmed no difference in these mechanical properties after the more aggressive original de-ep protocol.[16]

The cellular proliferation assay suggests that the new protocol has no negative effects on metabolism and growth of the BEAS-2B cells used for re-ep. This suggests that ECM scaffolds created using the new de-

ep protocol can support epithelial cell attachment and viability during re-ep, allowing for the creation of chimeric allografts.

This proof-of-concept study is not without limitations. To longitudinally measure chondrocyte survival, we incubated the de-epithelialized trachea in static Dulbecco's Modified Eagle Medium (DMEM) to simulate implantation of the grafts. This does not fully recapitulate the complex cell-environment interactions present *in vivo*. Therefore, conclusions regarding chondrocyte viability will need to be validated in a bioreactor environment that simulates nutrient perfusion, hydrodynamic stimuli, and mechanical stimuli.[26,27] The current study did evaluate chondrocyte viability of de-epithelialized trachea after a 7-day re-ep in a double-chamber bioreactor, yielding a percentage viability of 63% over 7-days. This result is promising given previous studies demonstrating that a 50% chondrocyte viability was associated with successful tracheal transplantation in dogs, with no lethal stenosis.[28] However, future studies should be conducted with a larger number of replicates.

In conclusion, we introduce a new de-ep protocol with improved short-term chondrocyte viability. The results of this study have indicated that improvements in the protocol can still be made. However, the data presented sheds light on the potential mechanism of chondrocyte death during and after de-ep.

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FIGURES AND TABLES.

Figure 1. The perfusion circuitry designed for the original de-ep protocol. Order of perfusion is numbered from 1-4 and corresponds to the solutions in table 1.

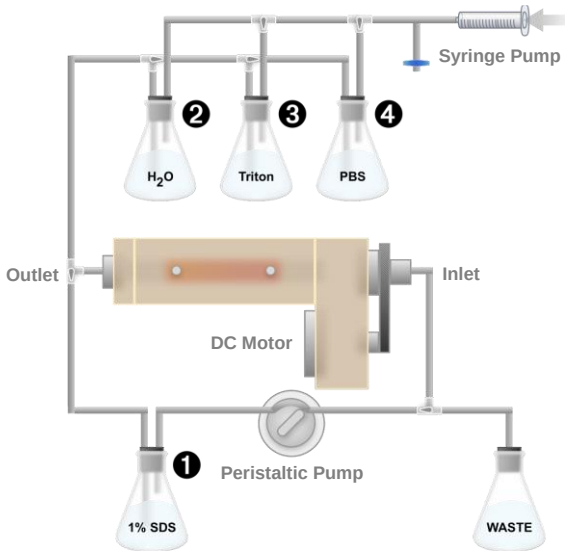


Figure 2. The perfusion circuitry designed for the new de-ep protocol. Order of perfusion is numbered from 1-5 and corresponds to the solutions in table 2.

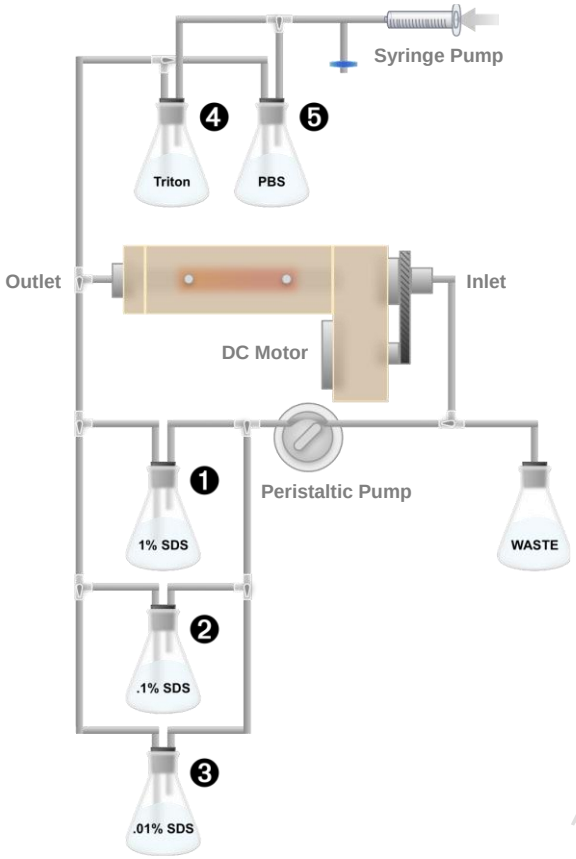


Figure 3. Appearance of the bioreactor with lid removed. Trachea is visible, surrounded by DMEM.

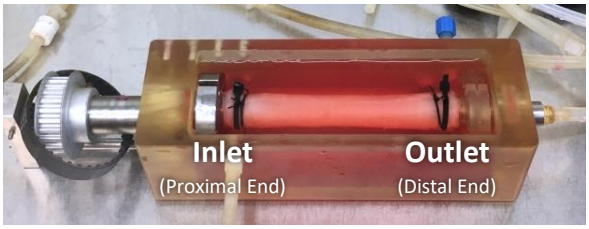


Figure 4. Timepoints for live/dead staining and histology used in this study.

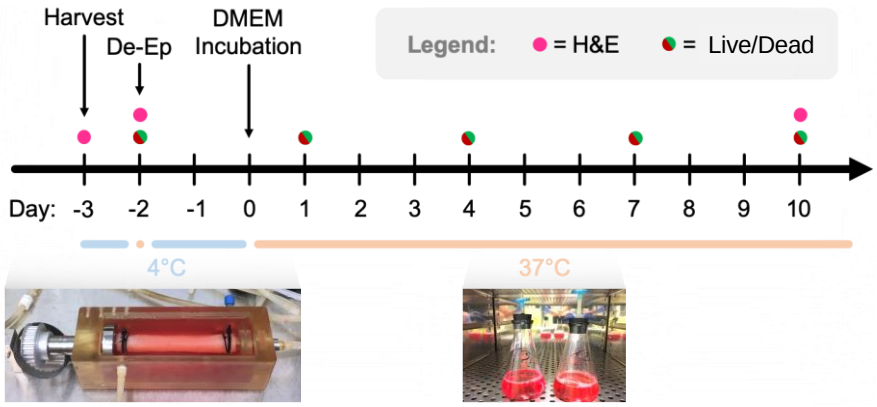


Figure 5. Chondrocyte viability following de-epithelialization and 10-day incubation in static media. Statistically significant differences as determined by a two-way ANOVA with Tukey's *post hoc* multiple comparisons test are indicated. P-values given as: <0.0332 = *, <0.0021 = **, <0.0002 = ***, <0.0001 = ****

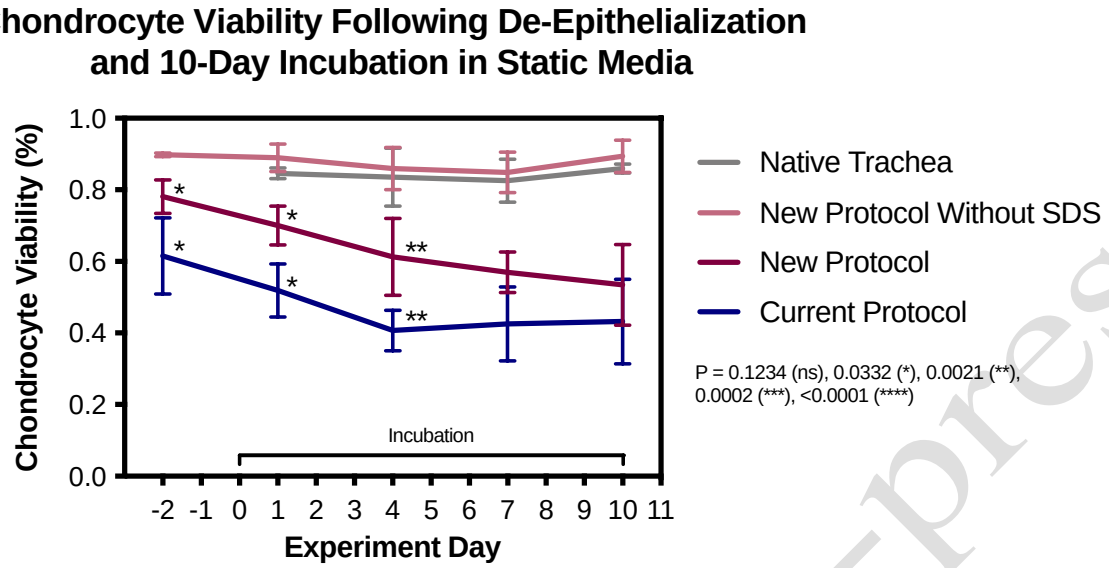


Figure 6. Chondrocyte viability in new protocol, original/current protocol, and a native trachea control on day 10 of static incubation. Confocal microscopy images depicting calcein-AM for live (green) and ethidium homodimer-1 for dead (red) cells in cross-sections of cartilage rings (marked as the area within the white dotted line).

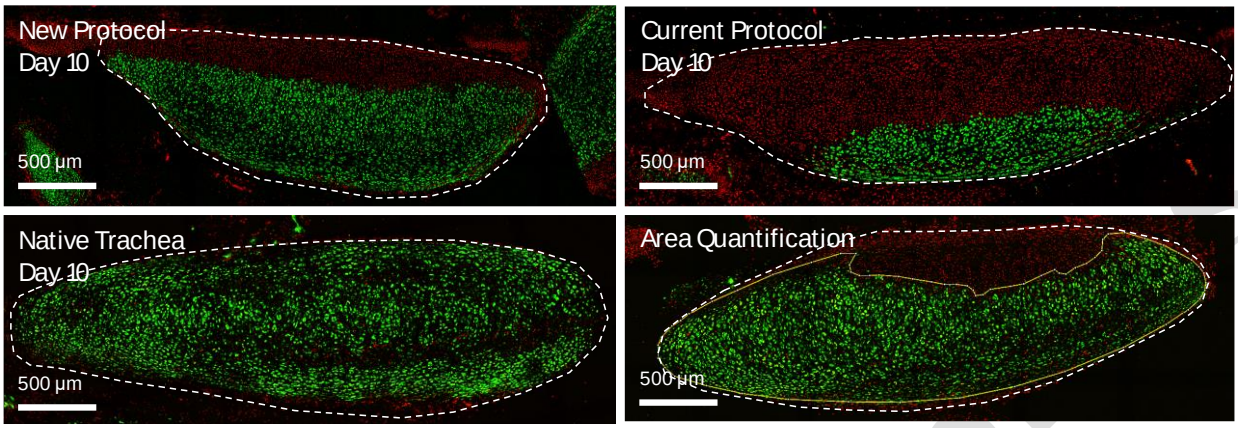


Figure 7. H&E of native trachea; trachea processed with the original/current de-ep protocol; and trachea processed with the new de-ep protocol. The lumen (L), epithelium (E), submucosa (SM), acinar glands (AG) and hyaline cartilage (HC) are labelled.

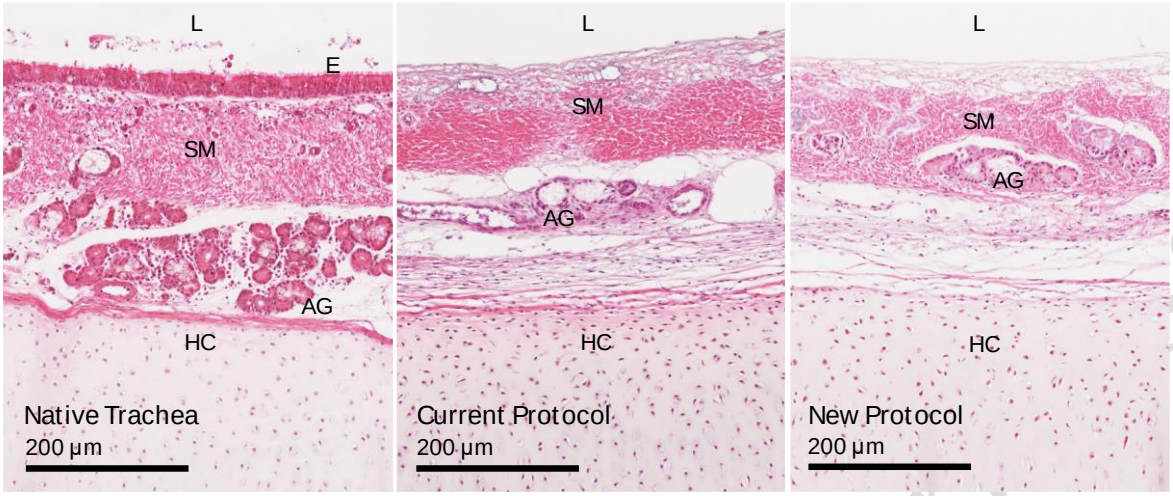


Figure 8. Masson's trichrome stain of native trachea; trachea processed with the original/current de-ep protocol; and trachea processed with the new de-ep protocol. 10x and 60x magnifications are shown in the top and bottom rows respectively.

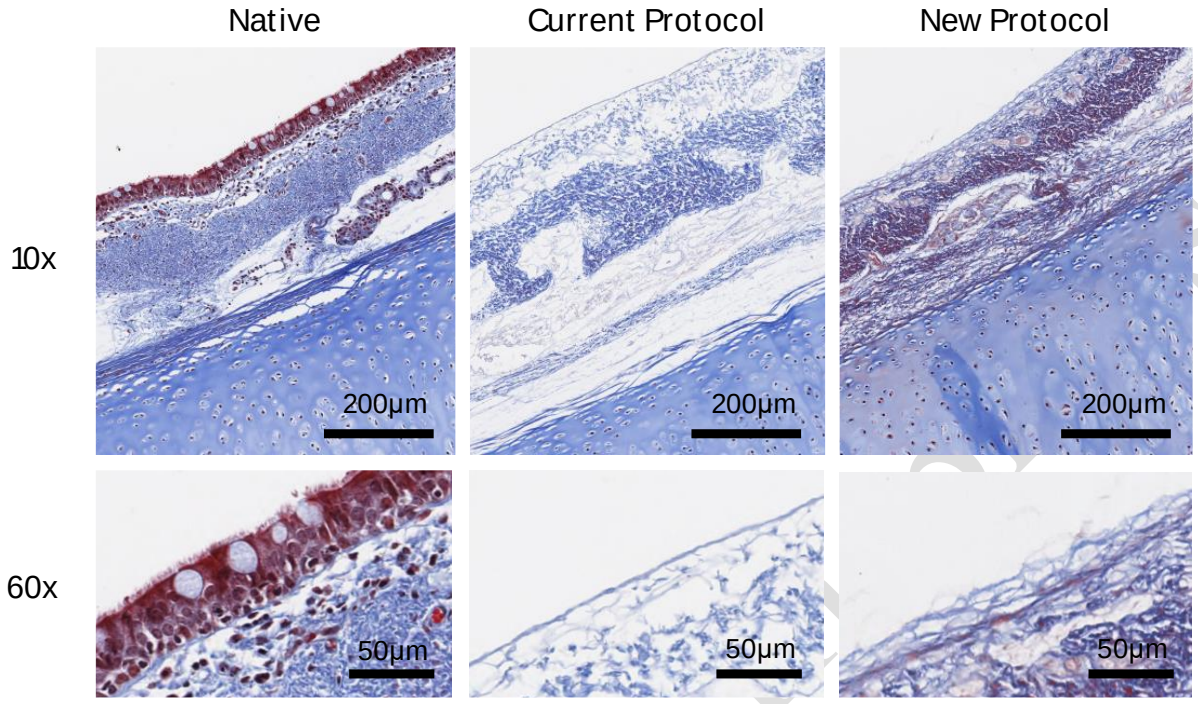


Figure 9. Verhoeff's elastin stain of native trachea; trachea processed with the original/current de-ep protocol; and trachea processed with the new de-ep protocol. 10x and 60x magnifications are shown in the top and bottom rows respectively.

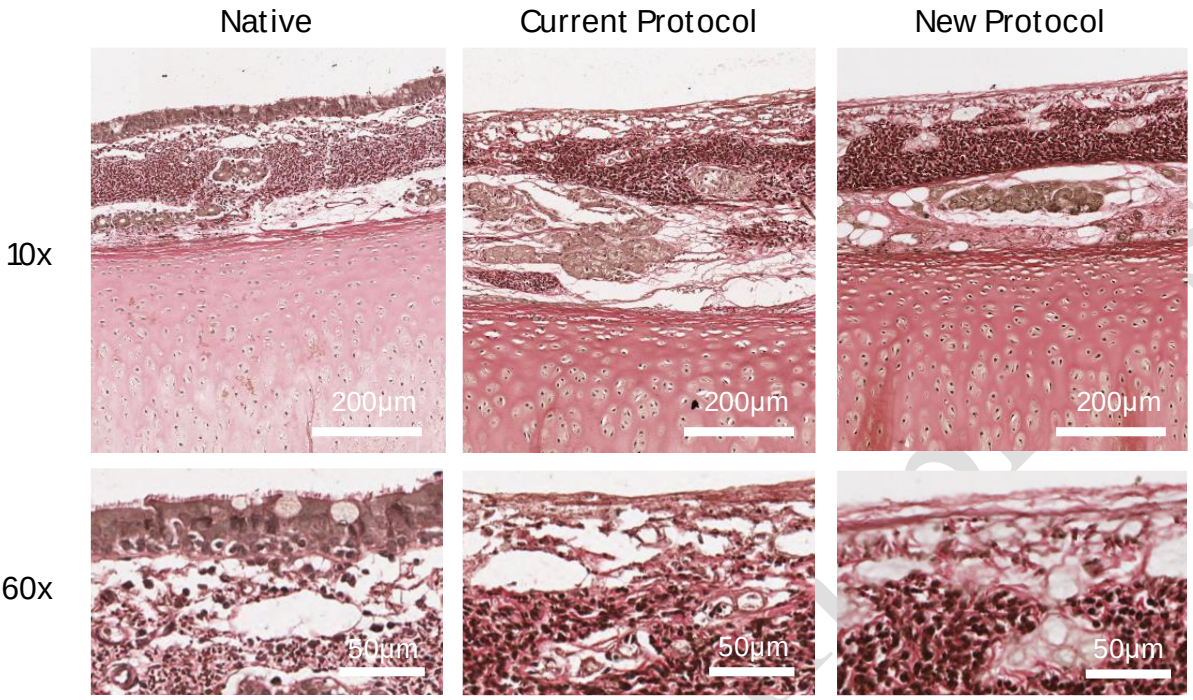


Figure 10. Alcian blue stain of native trachea; trachea processed with the original/current de-ep protocol; and trachea processed with the new de-ep protocol. 10x and 60x magnifications are shown in the top and bottom rows respectively.

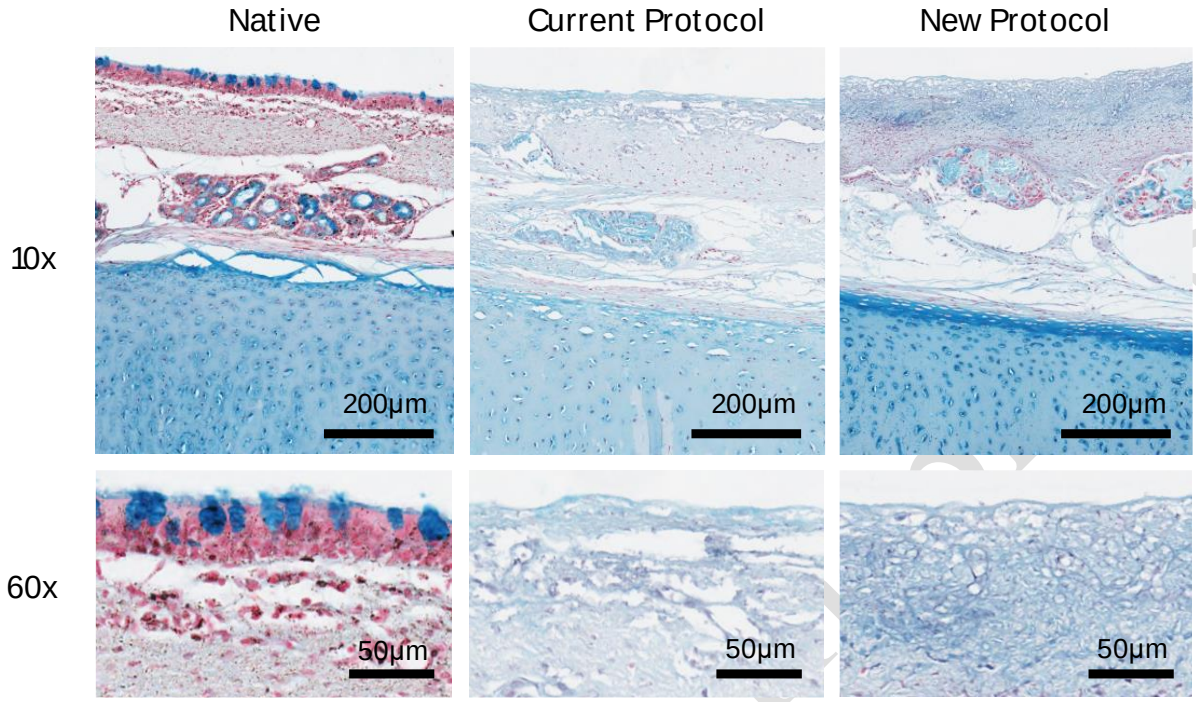
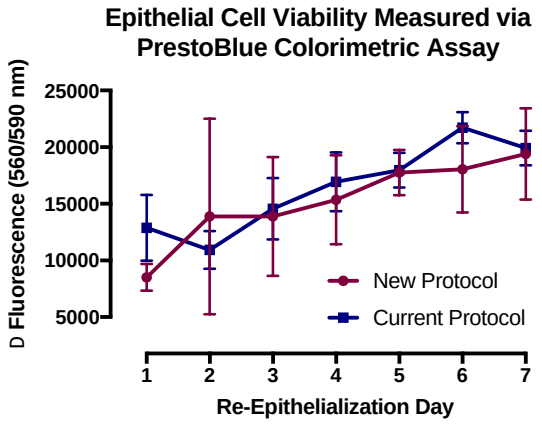


Figure 11. Growth curves of BEAS-2B on the new and old protocol's scaffolds over seven-day re-epithelialization period.



1 **Table 1.** Original de-epithelialization protocol

Step	Reagents*	Time	Vol. (mL)	pH	Temp. (°C)
1†	1% SDS	3 hr	75	7.4	37
2†	ddH_2O	30 min	140	7.4	37
4‡	1% Triton	30 min	140	7.4	37
5‡	DPBS (-/-)	30 min	140	7.4	37

* Reagents inside trachea (Lumen). Outside the trachea, DMEM with 10% FBS + 1% Pen/Strep solution remains circulating

† De-epithelialization process – pulsatile perfusion

‡ Washing steps – continuous perfusion

2

1 **Table 2.** New de-epithelialization protocol

Step	Reagents*	Time	Vol. (mL)	pH	Temp. (°C)
1†	1% SDS	1 hr	75	7.4	37
2†	0.1% SDS	1 hr	75	7.4	37
3†	0.01% SDS	1 hr	75	7.4	37
4‡	1% Triton	30 min	140	7.4	37
5‡	DPBS (-/-)	30 min	140	7.4	37

* Reagents inside trachea (Lumen). Outside the trachea, DMEM with 10% FBS + 1% Pen/Strep solution remains circulating

† De-epithelialization process – pulsatile perfusion

‡ Washing steps – continuous perfusion

2

Table 3. Control – New de-epithelialization protocol without SDS. Identical conditions as new protocol, except perfused with DPBS instead of SDS.

Step	Reagents*	Time	Vol. (mL)	pH	Temp. (°C)
1†	1% DPBS	1 hr	75	7.4	37
2†	1% DPBS	1 hr	75	7.4	37
3†	1% DPBS	1 hr	75	7.4	37
4‡	1% Triton	30 min	140	7.4	37
5‡	DPBS (-/-)	30 min	140	7.4	37

* Reagents inside trachea (Lumen). Outside the trachea, DMEM with 10% FBS + 1% Pen/Strep solution remains circulating

† De-epithelialization process – pulsatile perfusion

‡ Washing steps – continuous perfusion