# EFFECTS OF FREEZING/THAWING ON THE MECHANICAL PROPERTIES OF DECELLULARIZED LUNGS

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Running title: Decellularized lung mechanics after freezing/thawing.

Sources of support: This work was supported in part by the Spanish Ministry of Economy and Competitiveness (SAF2011-22576, FIS-PI11/00089) and by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (Research Productivity modality 307618/2010-2). Paula Naomi Nonaka has a fellowship (2012/04052-2) from Fundação de Amparo à Pesquisa do Estado de São Paulo.

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1002/jbm.a.34708

**Abstract**

Lung bioengineering based on decellularized organ scaffolds is a potential alternative for transplantation. Freezing/thawing, a usual procedure in organ decellularization and storage could modify the mechanical properties of the lung scaffold and reduce the performance of the bioengineered lung when subjected to the physiological inflation-deflation breathing cycles. The aim of this study was to determine the effects of repeated freezing/thawing on the mechanical properties of decellularized lungs in the physiological pressure-volume regime associated with normal ventilation. ~~Fifthteen~~ Fifteen mice lungs (C57BL/6) were decellularized using a conventional protocol not involving organ freezing and based on sodium dodecyl sulfate detergent. Subsequently, the mechanical properties of the acellular lungs were measured before and after subjecting them to 3 consecutive cycles of freezing/thawing. The resistance ($R_L$) and elastance ($E_L$) of the decellularized lungs were computed by linear regression fitting of the recorded signals (tracheal pressure, flow and volume) during mechanical ventilation. $R_L$ was not significantly modified by freezing-thawing: from 0.88±0.37 to 0.90±0.38 $cmH_2O \cdot s \cdot mL^{-1}$ (mean±SE). $E_L$ slightly increased from 64.4±11.1 to 73.0±16.3 $cmH_2O \cdot mL^{-1}$ after the three freeze-thaw cycles (p=0.0013). In conclusion, the freezing/thawing process that is commonly used for both organ decellularization and storage induces only minor changes in the ventilation mechanical properties of the organ scaffold.

**Keywords:** lung decellularization, organ scaffold, freezing/thawing, mechanical ventilation, elastance, lung bioengineering.

## INTRODUCTION

Organ transplantation is currently the last-resort therapeutical intervention for diseases in which the organ function is irreversibly injured. However, the scarcity of available viable organs and the lack of compatibility between donor and recipient human antigens reduce the success rate of transplantations. The problem of achieving successful organ transplantation is particularly difficult in patients with advanced respiratory diseases such as chronic obstructive pulmonary disease, lung fibrosis or pulmonary hypertension. Indeed, in current clinical practice only 50% of patients survive for 5 years after lung transplantation.[1]

In an attempt to find a new source of potential viable organs for transplantation, recent research approaches have focused on organ bioengineering.[2-5] Specifically, the underlying concept is the decellularization of a whole organ from a donor in order to obtain an scaffold - which keeps the anatomical 3D structure intact and is freed from genetic material of the donor - to be used as a biomimetic platform for the fabrication of a new organ by seeding the acellular scaffold with new cells, preferably ones with the genetic background of the organ recipient.[6,7]

Different procedures have been described in the literature to decellularize whole organs such as kidney, liver, heart and lung.[2-4,8] Most of the proposed procedures consist of a first treatment to break-down the integrity of the cells inside the organ, for instance by freezing/thawing, and a second treatment, based on different types of detergents, to extract the cellular material from inside the organ, leaving just the extra-cellular scaffold.

Freezing/thawing is a thermal process that seems necessary for lung bioengineering in future clinical applications. Indeed, whereas in research labs it is easy

to start the complex process of decellularization immediately after animal sacrifice and organ extraction, it would be generally impossible to perform the organ decellularization at the – sometimes remote and not specifically equipped – hospital where the donor is. Simply freezing the organ after extraction from the donor seems to be the most straightforward way to safely transport it to a center with the facilities required for decellularization. Moreover, regardless of whether freezing/thawing is involved as a first step in decellularization, it seems that freezing (and subsequent thawing) would be the most adequate way to store already decellularized organs until they are used for a further application.

Several authors have reported that freezing/thawing could have an impact on the viscoelastic properties of tissues, mainly as a result of the effect of ice formation within the scaffold biomaterials.[9-11] The results reported so far do not provide conclusions with general validity since the modifications induced by freezing/thawing in tissue mechanics depend on the organ specificity.[11-14] In particular, no data are available on the effects of freezing/thawing on the mechanical properties of the lung scaffold. This information is relevant to the bioengineering of this organ since, unlike other organs that remain essentially static, the physiological function of the lungs is based on their continuous mechanical changes associated with breathing. Therefore, the aim of this study was to measure the effects of repeated freezing/thawing – as described for organ decellularization[2] - on the mechanical properties of the lung scaffold in the physiological pressure-volume regime associated with normal ventilation. To this end, mouse lungs were decellularized at room temperature and their resistance and elastance were measured before and after cyclic freezing/thawing.

## METHODS

### Lung decellularization

This study was approved by the Ethical Committee for Animal Research of the University of Barcelona. Twenty-four female C57BL/6 mice, 7-8 weeks old (mean weight of 20±1.56 g, 17-22 g range) were intraperitoneally anesthetized with urethane (1 mg/kg) and sacrificed by exsanguination. The lungs and trachea were excised and kept in phosphate-buffered saline (PBS) at 4°C, for a maximum time of 30 minutes, and cleaned to remove any attached esophageal, lymphatic, and connective tissues. The lungs were then submitted to 6-8 washes with 2 mL of PBS instilled by the trachea, followed by 2.5 mL of deionized water, and then treated with tracheal instillation of 2.5 mL of 1% sodium dodecyl sulfate (SDS) detergent. The lungs were subsequently kept in agitation for 24 hours at room temperature in a 50 ml polystyrene conical tube with 3 mL of 1% SDS. The lungs were then rinsed again with 2 mL of PBS and maintained in 3 mL PBS in agitation for 24 hours to finish the process of obtaining acellular lung scaffolds.

### Decellularization assessment

Nine of the decellularized lungs were randomly selected to confirm correct decellularization and to assess the extracellular matrix components. The acellular lungs were divided into their lobes and fixed by bronchial infusion of a 3:1 ratio mixture of Optimal Cutting Temperature compound (OCT, Sakura) and PBS and stored at a -80ºC freezer. Subsequently, cryosections (10-15 µm) of frozen acellular lung samples were obtained using a cryomicrotome (Thermo Scientific, HM 560 CryoStar). To verify the

absence of cell DNA after the process of decellularization, 4',6-diamidino-2-phenylindole (DAPI) fluorescence staining was used. The cryosections were rinsed with PBS to remove the OCT and then maintained for 10 minutes with 1µg/mL DAPI (Sigma) solution for staining. Extracellular matrix proteins in the decellularized lungs were assessed by immunofluorescent staining of the cryosections. To this end, they were washed with PBS to remove the OCT and were fixed with 4% paraformaldehyde for 30 minutes at room temperature. A blocking solution was then applied for 1 h (1% BSA, 6% fetal bovine serum, 0.5% triton in tris-buffered solution) and kept under incubation overnight. The primary antibodies were detected by using appropriate secondary antibodies. The following antibodies were used: anti-collagen-I (Abcam), anti-laminin (Sigma), anti-collagen-IV (Santa Cruz), anti-(tropo)elastin (Elastin Products Company). Images were taken using a Nikon Eclipse Ti fluorescent microscope. Glycosaminoglycans (GAGs) in the decellularized lung cryosections were assessed by staining with alcian blue solution (Alcian blues, 8GX; acetic acid 3% solution) for 30 minutes and dehydrated.

**Mechanical ventilation of decellularized lungs**

The mechanical properties of the 15 acellular lungs were first measured immediately after decellularization. To characterize the pressure-volume relationship in mechanical conditions similar to those in physiologically normal breathing, the decellularized lungs were subjected to conventional mechanical ventilation. To this end, the acellular lungs were tracheally intubated using a 18-gauge metallic cannula, suspended vertically by gravity and placed within a chamber similar to the ones typically used in ex vivo lung preparations (32°C and 100% relative humidity). A

pneumotachograph (range ±20 ml/s) was connected to the inlet of the cannula to measure tracheal flow (V') by sensing the pressure drop across the pnemotachograph with a differential pressure transducer (DC001NDC4, range ±2.5 $cmH_2O$). Tracheal pressure ($P_{tr}$) was mearured by connecting a pressure transducer (176PC14HD2, ±35 $cmH_2O$) on a side port placed between the pneumotachograph and the cannula. The inlet of the pneumotacograph was then connected to the Y piece of a volumetric mechanical ventilator designed for the artificial ventilation of rodents.[15] The decellularized lungs were subjected to conventional ventilation with a quasi-sinusoidal flow pattern with a tidal volume of 10 ml per kg of mouse body weight, a frequency of 60 breaths/min and a positive end expiratory pressure (PEEP) of 2 $cmH_2O$, to counteract the absence of the physiological negative pleural pressure at rest. Flow and pressure signals from the transducers were analogically low-pass filtered (Butterworth, 8 poles, 32 Hz), sampled at a rate of 100 Hz (PCI-6036, National Instruments) through a custom monitoring and recording application (LabView).

**Measurement of respiratory resistance and elastance in decellularized lungs**

Acellular ~~L~~lung resistance and elastance were computed from the signals recorded during mechanical ventilation. A first step was to compute the volume signal (V) by digital integration of the flow signal (V'). Secondly, the tracheal pressure ($P_{tr}$) signal was corrected by subtracting the pressure drop ($P_{can}$) caused by the non-linear resistance of the intubation cannula, which had been previously calibrated and characterized ($P_{can}= K_1 \cdot V' + K_2 \cdot |V'| \cdot V'$, where $K_1$ and $K_2$ are linear and non-linear parameters of the Rohrer model). In a subsequent step, the effective ~~lung~~ resistance

($R_L$) and elastance ($E_L$) of the decellularized lung were computed by linear regression fitting of the recorded signals $P_{tr}$, V' and V to the conventional respiratory mechanics model $P_{tr} = P_o + E_L \cdot V + R_L \cdot V'$, where $P_o$ is a parameter to account for the external PEEP applied by the ventilator.[16] For each decellularized lung, $R_L$ and $E_L$ were computed from data including five breathing cycles. To assess acellular lung viscoleasticity and homogeneity, static and dynamic elastances were also measured by means of end-inspiratory airway occlusions achieved by pushing the corresponding control button of the mechanical ventilator. After an end-inspiratory occlusion, there is a fast initial drop in acellular lung pressure ($\Delta P_1$) from the preocclusion value down to an inflection point (with pressure $P_i$), followed by a slow pressure decay ($\Delta P_2$) until a plateau pressure ($P_{el}$) corresponding to the elastic recoil pressure of the decellularized lung is reached. Whereas $\Delta P_1$ is associated with pressure dissipated against pulmonary resistance, $\Delta P_2$ reflects tissue viscoelastic properties or pendeluft. Accordingly, acellular lung static elastance ($E_{L,st}$) was computed as the plateau pressure ($P_{el}$) recorded after 5 s of occlusion divided by the tidal volume, and lung dynamic elastance ($E_{L,dyn}$) was computed by dividing the inflection point pressure ($P_i$) by the tidal volume.[17-19] For each decellularized lung, $E_{L,st}$ and $E_{L,dyn}$ were obtained as the means from 5 end-inspiratory occlusions, each one carried out after one minute of normal mechanical ventilation.

**Freezing and thawing decellularized lungs**

After measuring lung scaffold mechanics immediately after decellularization, the acellular lungs were de-intubated, placed in a 15 mL polystyrene conical tube with 3 mL PBS and subjected to 3 consecutive cycles of freezing/thawing, each one consisting of

the following steps: room temperature, -20°C, -80°C, -20°C and room temperature., ~~each temperature being maintained for at least 2 h.~~ Each step at room temperature and at -20ºC lasted 2 h and each -80ºC step was kept overnight (>12 h). After the freezing/thawing cycles were finished, the mechanical properties of the acellular lungs were measured in exactly the same way as immediately after decellularization.

**Statistical analysis**

Comparisons between the values of $R_L$, $E_L$, $E_{L,st}$ and $E_{L,dyn}$ measured before and after the freezing/thawing process for each decellularized lung were carried out by paired *t*-tests. Linear regressions between variable were also computed. The p value was considered statistically significant at the 5% level.

**RESULTS**

Examples of intact and decellularizad mouse lungs are shown in Figure 1. As illustrated by Figure 2, the acellular lungs were freed from cells (DAPI staining, Figure 2) and the lung scaffold retained relevant extracellular proteins (Collagen I and IV, elastin, laminin) and GAGs.

Figure 3 (top) shows an example of the excellent agreement between actual tracheal pressure during mechanical ventilation of an acellular lung and the pressure corresponding to the model fitting to compute $R_L$ and $E_L$, showing the adequacy of this simple linear respiratory modeling. A representative example of the end-inspiratory occlusion measurements in a decellularized lung is shown in Figure 3 (bottom).

Remarkably, the flatness of the pressure plateau indicates the absence of air leaks, reflecting the fact that decellularization maintained the structural integrity of the lung.

Figure 4 depicts the values of $R_L$ and $E_L$ before and after application of the freezing/thawing cycles. The correlation plots show the good concordance between pre- and post-freezing/thawing for each individual decellualrized lung. Whereas no significant differences were observed in $R_L$, $E_L$ significantly increased after freezing/thawing. Nevertheless, this increase was quite small (13.5% on average). Similarly, Figure 5 shows that, for each acellular lung, both $E_{L,st}$ and $E_{L,dyn}$ before and after the repeated low-temperature challenge were very well correlated. On average, and in agreement with $E_L$ data, $E_{L,st}$ and $E_{L,dyn}$, increased modestly, although significantly, (by 15.7% and 15%, respectively).

## DISCUSSION

Freezing/thawing is a procedure commonly used in lung bioengineering, both for organ decellularization and for storing the acellular lung scaffold before recellularization. Given that it was unknown whether this specific thermal challenge could modify the relevant mechanical properties of the decellularized lung, we addressed this question and showed that cyclic freezing/thawing induces only minor changes in the resistance and elastance of the acellular lung.

We used an experimental approach specifically designed to assess the mechanical properties of decellularized lung scaffolds in dynamic conditions mimicking physiological breathing. Indeed, to determine the exclusive effects of freezing/thawing - i.e. avoiding the ones potentially caused by other steps in the decellularization process-

we studied the mechanical properties of acellular lungs obtained via a procedure carried out at room temperature, thereby avoiding any thermal challenge to the organ scaffold. We then measured the viscoelastic properties of these acellular scaffolds just before and after the freezing/thawing cycles. Acellular lung mechanics were also characterized under the physiological conditions of breathing. To this end, the organ scaffold was subjected to conventional mechanical ventilation to measure resistance and elastance, as usually undertaken in patients.[20,21]

The resistance and elastance values we obtained in decellularized lungs at conventional ventilation settings (breathing frequency, tidal volume and end-expiratory pressure) were consistent with the figures reported by other authors in acellular lungs subjected to other types of mechanical tests, such as quasi-static high-volume excursions or low-amplitude high-frequency forced oscillation.[4,22] Using our experimental approach, we were able to provide novel data on the relationship between static ($E_{L,st}$) and dynamic ($E_{L,dyn}$) elastance measured in acellular lungs during conventional ventilation (Figure 5). As expected for a viscoelastic system such as the acellular lung,[23,24] $E_{L,dyn}$ was greater than $E_{L,st}$. However, the relatively small difference between these two elastance parameters (on average, $E_{L,dyn}/E_{L,st}$ was 1.07 both, before and after freezing/thawing) suggests that the acellular lung has an almost pure elastic pressure-volume relationship and negligible inhomogeneity. This notion concurs with the excellent fitting achieved when the ventilation signals were interpreted using a $R_L$-$E_L$ model (Figure 3), and with the similar values obtained for $E_L$ and $E_{L,dyn}$ (Figures 4 and 5), suggesting that the simple resistance-elastance model commonly used to monitor intact lung mechanics (in both animals and patients) is also applicable to decellularized lungs.

Indeed, the $E_{L,dyn}/E_{L,st}$ values we found in acellular lungs were similar to those reported for intact mouse lungs in vivo, using the same measuring technique (ranging from 1.02 to 1.15).[19,25,26]

The effects of freezing/thawing on the elastic properties of biological samples have been studied in different kinds of intact tissues: vascular grafts, larynges, fat tissue, osteochondral dowels, mammary gland, tendons and bones.[9-12,14,27,28] Whereas in some cases the authors reported a slight decrease in stiffness after freezing/thawing, most reports on cryopreservation indicate no mechanical changes induced by freezing/thawing. The limited number of available reports evaluating the effect of applying subsequent cycles of freezing/thawing to intact tissues indicates that subjecting the sample to only one, or a few more, thermal cycles does not modify tissue elasticity.[13,27,28] In contrast with intact tissues, the information available on the mechanical effects of freezing/thawing in decellularized tissue scaffolds is scarce, with one work on pulmonary valves reporting a slight decrease in stiffness depending on the thermal protocol[29] and another report showing no mechanical differences induced by freezing/thawing in acellular arteries.[30] The novel results we obtained in decellularized lungs show a small increase in scaffold elastance after three slow freezing/thawing cycles. Although we did not investigate the specific cause, this small change in elastance could be attributable to small changes induced by the formation/melting of ice crystals[31] at a non-uniform rate,[32] within the scaffold fibers and their junctions, which are the elements determining the 3D bulk deformation of bronchi and alveoli during breathing volume changes. However, it should be noted that the 10-15% increase we observed in lung elastance would have no physiological implications in terms of the

performance of the lung scaffold when subjected to conventional ventilation during the recellularization process, since keeping a given level of ventilation would require only a minor 10-15% increase in inspiratory pressure.

In conclusion, the present study indicates that freezing/thawing has a ~~non-relevant~~ small effect on the ventilation properties of decellularized lung scaffolds, which favors the use of this thermal process for both lung decellularization and scaffold storage in lung bioengineering, which is a very promising application in regenerative medicine.[33]

**ACKNOWLEDGMENTS**

The authors wish to thank Miguel A. Rodríguez for his excellent technical support.

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## FIGURE LEGENDS

**Figure 1**. Representative example of an intact (left) and a decellularized (right) mouse lung.

**Figure 2**. Representative examples comparing sections of intact mice lungs (A-C and G-I) and decellularized mice lungs (D-F and J-L). Fluorescent DAPI staining (A, D, G and J) indicates that after decellularization (D and J) the lung scaffold did not contain cell DNA. Staining of collagen I (B and E) and IV (H and K), elastin (C and F), laminin (I and L) and glycosaminoglicans (intact lungs represented in image M and decellularized lungs in image N) show that these relevant extracellualar matrix components remained in the acellular lung scaffold.

**Figure 3**. Top: Tracheal pressure ($P_{tr}$) during mechanical ventilation of an acellular lung (solid line) and pressure corresponding to the fitting of a resistance-elastance lung model (dotted line). Bottom: Representative example of the pressure recorded during an end-inspiratory occlusion in a decellularized lung.

**Figure 4**. Effective elastance ($E_L$) and resistance ($R_L$) computed during the conventional mechanical ventilation of decellularized lungs before (black column) and after (grey column) cyclic freezing/thawing. Values are mean±SE. Asterisk indicates paired t-test with $p<0.05$. Solid line is the identity line, dashed line is the linear regression fitting. R is the regression coefficient ~~of linear regression~~.

**Figure 5**. Static ($E_{L,st}$) and dynamic ($E_{L,dyn}$) elastances computed and after an end-inspiratory occlusion maneuver in decellularized lungs before (black column) and after (grey column) cyclic freezing/thawing. Values are mean±SE. Asterisk indicates paired t-test with $p<0.05$. Solid line is the identity line, dashed line is the linear regression fitting. R is the regression coefficient ~~of linear regression~~.

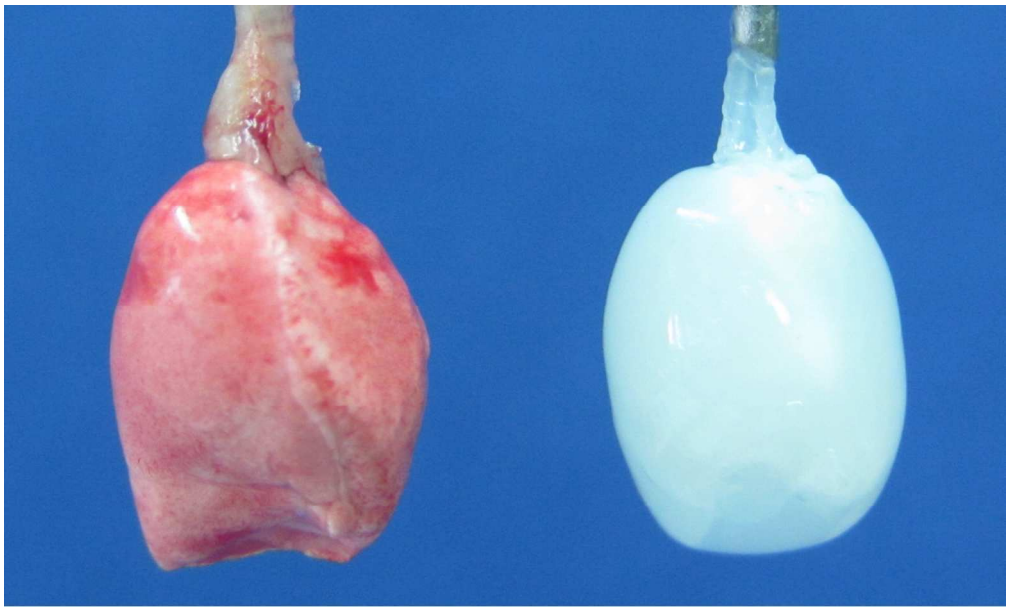

Figure 1. Representative example of an intact (left) and a decellularized (right) mouse lung.
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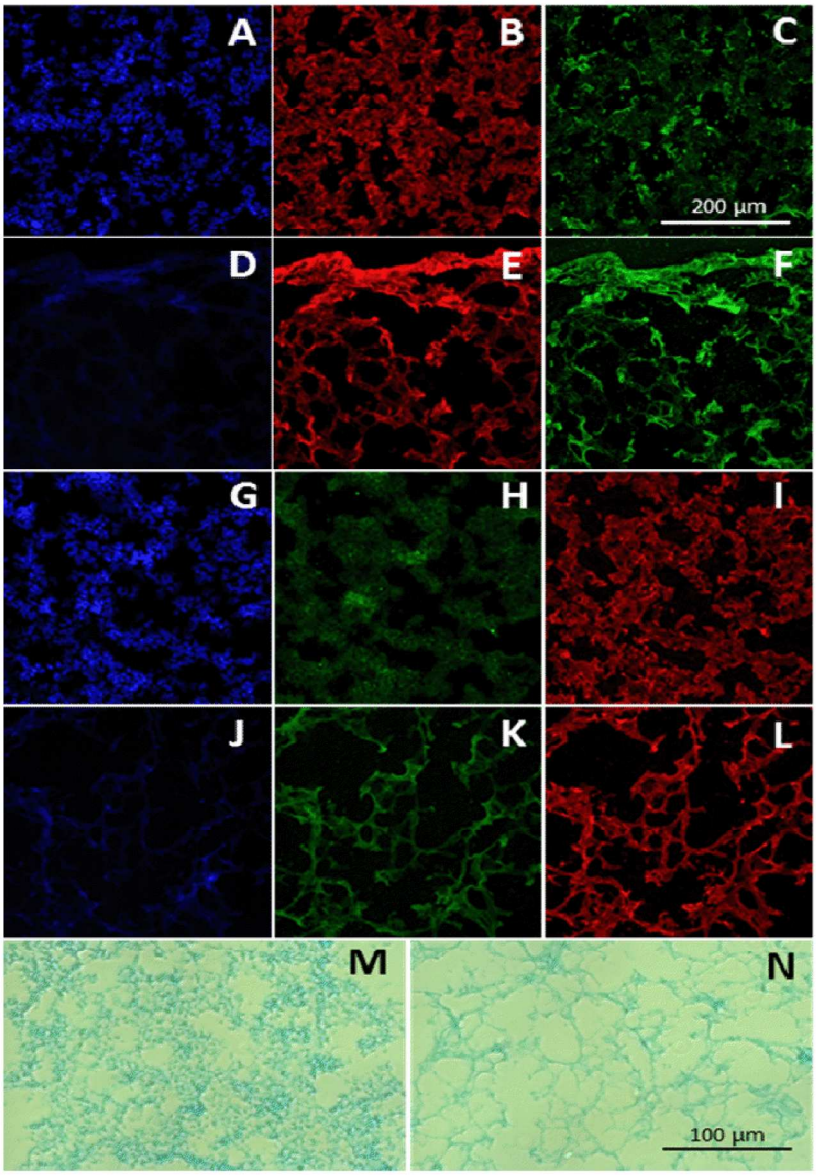


Figure 2. Representative examples comparing sections of intact mice lungs (A-C and G-I) and decellularized mice lungs (D-F and J-L). Fluorescent DAPI staining (A, D, G and J) indicates that after decellularization (D and J) the lung scaffold did not contain cell DNA. Staining of collagen I (B and E) and IV (H and K), elastin (C and F), laminin (I and L) and glycosaminoglicans (intact lungs represented in image M and decellularized lungs in image N) show that these relevant extracellualar matrix components remained in the acellular lung scaffold.
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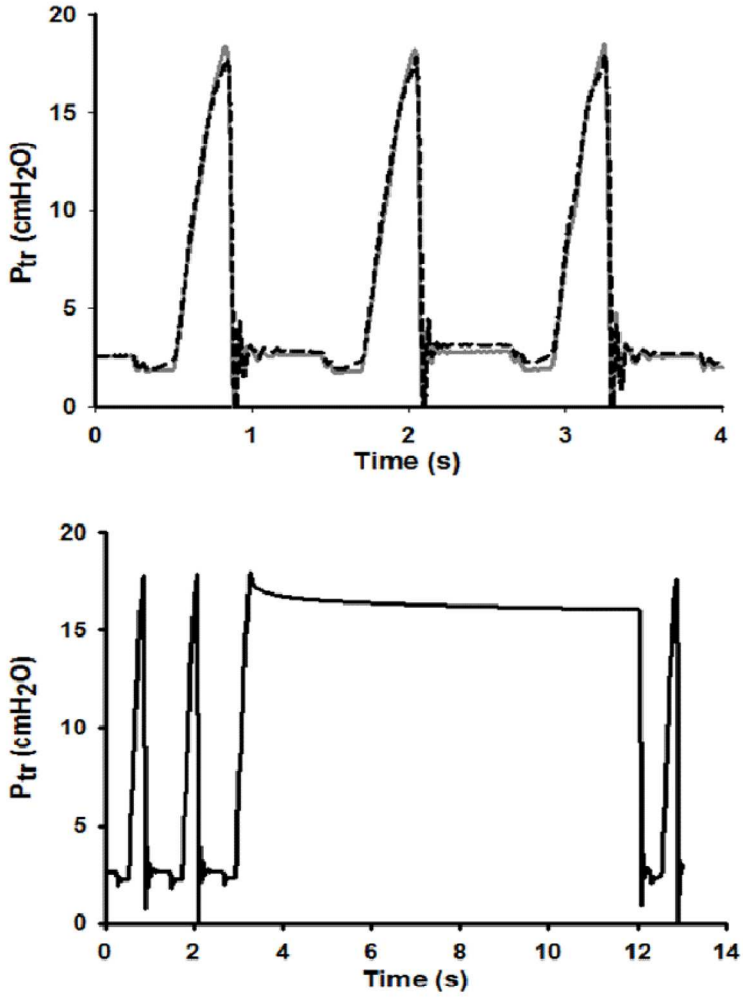


Figure 3. Top: Tracheal pressure (Ptr) during mechanical ventilation of an acellular lung (solid line) and pressure corresponding to the fitting of a resistance-elastance lung model (dotted line). Bottom: Representative example of the pressure recorded during an end-inspiratory occlusion in a decellularized lung.
127x177mm (300 x 300 DPI)

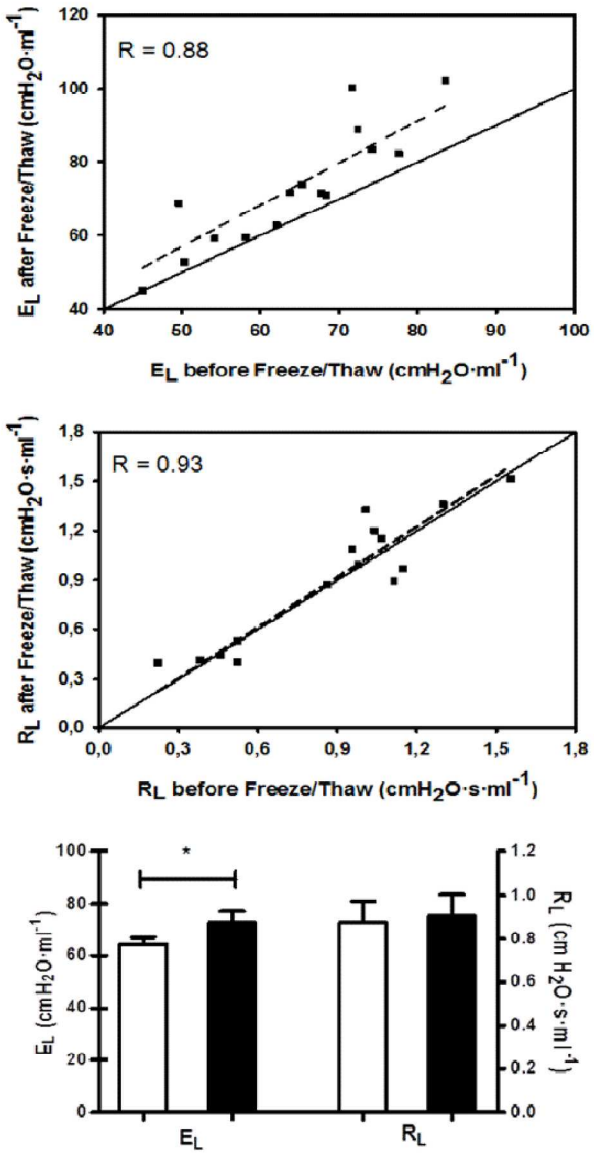


Figure 4. Effective elastance (EL) and resistance (RL) computed during the conventional mechanical ventilation of decellularized lungs before (black column) and after (grey column) cyclic freezing/thawing. Values are mean±SE. Asterisk indicates paired t-test with $p<0.05$. Solid line is the identity line, dashed line is the linear regression fitting. R is the regression coefficient.
127x177mm (300 x 300 DPI)

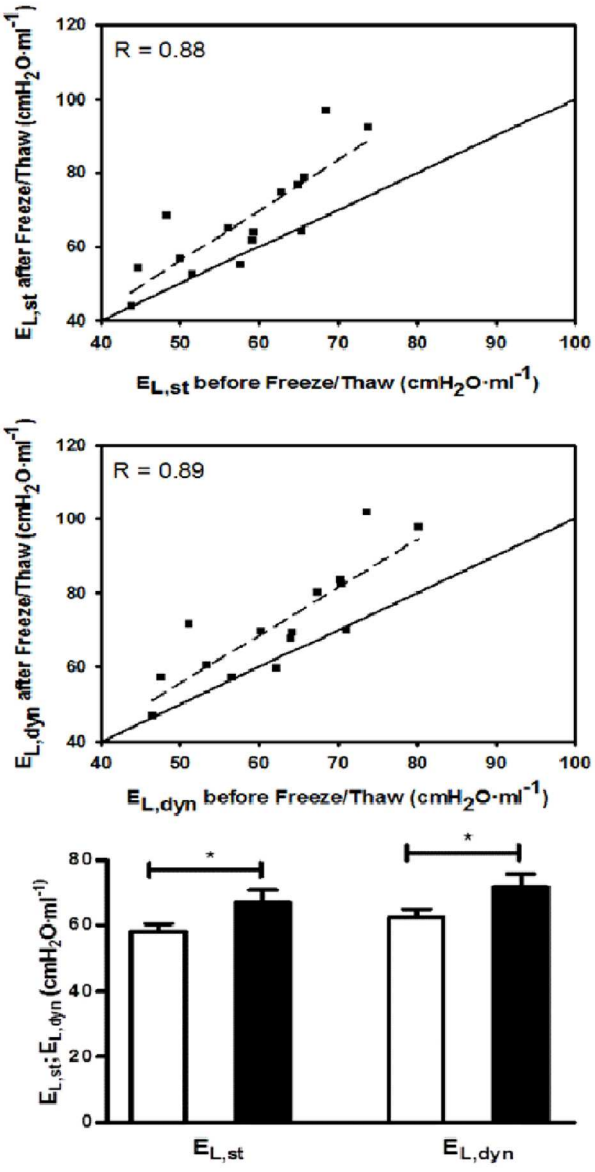


Figure 5. Static (EL,st) and dynamic (EL,dyn) elastances computed and after an end-inspiratory occlusion maneuver in decellularized lungs before (black column) and after (grey column) cyclic freezing/thawing. Values are mean±SE. Asterisk indicates paired t-test with $p<0.05$. Solid line is the identity line, dashed line is the linear regression fitting. R is the regression coefficient.
101x152mm (300 x 300 DPI)