Scanning Microscopy

Volume 1 | Number 2 Article 39

2-23-1987

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Nelson, Alan C. (1987) "Study of Rat Lung Alveoli using Corrosion Casting and Freeze Fracture Methods Coupled with Digital Image Analysis," *Scanning Microscopy*: Vol. 1 : No. 2 , Article 39.
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Scanning Microscopy, Vol. 1, No. 2, 1987 (Pages 817-822)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA
0891-7035/87$3.00+.00

STUDY OF RAT LUNG ALVEOLI USING CORROSION CASTING AND FREEZE FRACTURE METHODS COUPLED WITH DIGITAL IMAGE ANALYSIS

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(Received for publication September 19, 1986, and in revised form February 23, 1987)

Abstract

Relative areas and volumes can be estimated from vascular corrosion casts of rat lung alveoli using a calibration obtained from bulk frozen hydrated tissue. These morphometric measurements are roughly independent of the shrinkage and distortion artifacts known to arise in the corrosion casting procedure. Digital image processing of the SEM micrographs is employed to facilitate the measurement of casts and frozen tissue.

The vascular corrosion casting technique is modified also to permit successful casting of alveolar air passages. The modified technique produces faithful casts of dead-ended luminal structures where continuous perfusion of casting medium into the tissue is not possible. The casts of alveolar air passages and their corresponding vasculature are compared to determine the volume of a single alveolus.

By utilizing the calibrated measurements of lung areas and volumes, an estimate of lung vascular surface area per unit volume is obtained. This number, when multiplied by the respiratory tidal volume of the rat, may represent the total lung vascular surface area available for physiological gas exchange during normal respiration.

Key Words: Alveoli, Corrosion Casting, Freeze Fracture, Surface Area, Vasculature.

Introduction

The corrosion casting technique is a powerful tool in the analysis of luminal spaces within biological tissue. Generally, the technique is employed to provide an opportunity for three-dimensional visualization of luminal structures under the scanning electron microscope. While it would be a formidable task to make absolute spatial measurements of three-dimensional casts which have undergone uncontrolled distortion and shrinkage (Lametschwandtner et al., 1984), it may be possible to make relative measurements of areas and volumes under some circumstances. With the aid of digital image processing of SEM micrographs, these measurements can be made straightforwardly (Nelson, 1986) and may provide insight into the physiology of the system under examination.

The luminal structure of the blood circulatory system has been investigated widely (Lametschwandtner et al., 1984). The blood circulatory system has the property that it can circulate fluid freely, and therefore, the injection of casting material to fill the vessel lumens can be readily accomplished as has been demonstrated previously for rat lung vasculature (Hijiya and Okada, 1978; Ohtani, 1980) and for avian lung vasculature (Nowell et al., 1972).

However, some biological luminal spaces do not possess convenient circulatory pathways and, instead, are dead-ended. Examples of these systems include the airways of the lung (Dilly, 1984), and the urinary ducts of the kidney. These dead-ended luminal systems are a challenge for the corrosion casting technique, since pockets of fluid within the luminal system can easily block the flow of casting polymer resulting in only partial casting (Murakami, 1978).

For a morphometric analysis of lung alveoli it would be useful to have casts of both the alveolar capillary network and the alveolar airway. While casting of the airway presents special difficulties, we were able to produce alveolar vascular casts and airway casts separately or together in the same tissue; thus a more thorough understanding of the three-dimensional character of alveoli was possible.

To provide calibration for the measurement of casts, we compared the casts of alveolar structures with those observed in a preparation of frozen hydrated lung tissue which had been fractured open, sublimed and analyzed under SEM in the bulk frozen state (Echlin, 1978). By correlating structures in the

frozen and cast preparations it was possible to calculate the lung alveolar surface area per unit alveolar volume.

Materials and Methods

Wistar-Lewis albino male rats of about 200g body weight were used throughout this study. Anesthesia was induced with an intraperitoneal injection of pentobarbital sodium (3mg/100g). To reduce the possibility of thrombus formation, 0.05ml lab grade heparin sodium (Fisher Scientific Co., Fair Lawn, NJ) was injected into the saphenous vein and was allowed to circulate for one minute. The first incision was made near the central anterior diaphragm using surgical scissors. Two lateral cuts of about 3cm each were made, then the left and right rib cage bones were snipped and the whole anterior rib cage was lifted to expose the heart and lungs. We made no attempt to control bleeding.

The left ventricle of the heart was cannulated and perfused with heparinized saline (0.85% saline with KC1 to stop the heart beating) through a gravity drip line regulated to about 5 drops per second at 40cm height. A snip was made in the right atrium of the heart to drain excess perfusate, and the aorta was clamped with a hemostat near the abdominal region. Retrograde perfusion of the lungs was initiated and continued until the lung tissue appeared white, usually after about 400ml perfusate. The retrograde perfusion was used because of the relatively easy cannulation of the left ventricle.

Fixation was achieved by inserting a syringe in the rubber drip line and injecting 20ml of 0.25% glutaraldehyde in phosphate buffer (300mosM, pH 7.3) at room temperature after which the perfusion was temporarily stopped to allow glutaraldehyde to permeate the tissue. Within a few minutes the lung tissue had toughened as fixation occurred and after five minutes the perfusion was started again.

At this point one must decide whether to obtain only an airway cast, a vascular cast, or both. If both casts are desired, then the airway cast should always be made first. The casting material used in this work was blue Mercox (Ladd Research, Burlington, VT) mixed as 20g monomer and 0.4g initiator.

For a cast of lung airways the lungs were inflated with helium gas administered through a tube pressed over the nose and mouth. While the lungs were inflated the trachea was sutured shut above the bronchial branches. Mercox polymer in a 10 ml syringe was injected under hand pressure into the isolated trachea to fill the lungs. The lung tissue acquired a blue hue as the polymer filled the airways. About 10 min of hardening time was then required.

The inflated lung vasculature was cast either with or without prior casting of the airways; the procedure was the same in either case. The left ventricle cannula to which the perfusion line attaches was used for Mercox injection simply by replacing the drip line with a 10ml syringe filled with Mercox. The polymer was injected under hand pressure, and as vascular filling occurred, the lung surface became blue in color. Polymer was injected until excess injectate flowed from the snip in the right atrium of the heart. About 10 min was allowed for hardening of the cast.

The casted lungs were removed from the animal, stored in water for one day to cure the cast, then placed in 4 normal KOH at room temperature for maceration for a period of one week. The casts were then prepared for SEM by dehydration in graded ethanol, air drying for one day in a desiccator followed by drying under 1 torr vacuum for 2 h, mounted on specimen stubs using silver colloid glue and sputter coated with gold. To obtain smaller pieces of lung cast, the original cast was simply pulled and broken apart to obtain more convenient pieces. SEM (ISI DS-130, Milpitas, CA) of the casts was performed at 20 kV in the secondary electron signal mode.

Frozen hydrated tissue was prepared by anesthetizing the rat, surgically removing the rib cage and inflating the lungs as before. After suturing the trachea, the living lung tissue was quickly cut from the animal and placed in liquid nitrogen slush until frozen solid. A cold knife was used to fracture the bulk tissue into smaller pieces which were loaded in the frozen state onto the SEM cold stage (SP-2000, Photometrics, Woburn, MA) for analysis. The bulk frozen tissue was not metal coated prior to SEM and was allowed to partially sublime by warming its surface in the vacuum of the electron microscope. SEM was performed on the bulk frozen hydrated lung tissue at 5 kV using the backscatter signal mode to avoid specimen charging artifacts.

Digital image processing was employed to obtain measurements from SEM micrographs of the percent vascular area of the alveolus wall and average alveolar volume. For measurement of area, the specimen alveolus cast was oriented under the SEM by eye such that the alveolar surface in the image was approximately perpendicular to the beam axis. The resulting micrograph was placed on a light stand and viewed with a video camera connected to a digitizing frame buffer (Datacube, Danvers, MA) which in turn was connected to a computer (VAX 11/750, DEC, Marlboro, MA). This hardware system has been described elsewhere (Nelson, 1986).

With all image pixel gray values that were greater than or equal to an adjustable threshold being set to one and all values below being set to zero, a binary image was created such that black (zero) represented non-vascular areas in the specimen and white (one) represented vascular areas. The ratio of the number of white pixels to the number of black plus white pixels was taken to be approximately equal to the proportion of vascular surface area on the specimen. The thresholds used for relative area assessment and for contouring were best set by eye through a user interactive front-end monitor and mouse driver (Summagraphics Corp., San Jose, CA).

Cast specimens of lung airways showed alveolar air sacs as roughly ellipsoid in shape. With digital contouring of the airway micrographs, the approximate perimeters of sacs were found. Air sac volumes were computed from measured major and minor axes of the elliptical air sac perimeter contours where it was assumed that the volume shape was ellipsoid.

Results

Cast specimens of rat lung were prepared from ten rats. While partial filling of Mercox and breakage of the fragile casts were frequent problems, regions of good cast were located in each lung preparation.

Figure 1 is a representative micrograph of the rat lung vascular cast showing the capillary network in several alveoli. To obtain these views of the inside of the alveoli, the casts were broken to reveal the interior. By aligning the specimen so that central areas of the alveoli were perpendicular to the SEM beam, we generated micrographs suitable for digitization and image processing. At magnifications of about 1000X we analyzed approximately 25% of the surface area of each alveolus. Digital processing of binary images of over 100 alveolar surfaces like those shown in Figure 1 gave a vascular surface area per unit alveolus surface area of 0.86 ± 0.05. In other words, 86% of the alveolus surface contains vasculature.

An airway cast is shown in Figure 2 where the central broncheolus is seen to branch into clusters of alveolar air sacs which vary in size. To calculate volume, our imaging software assumed only that the air sac shape was an ellipsoid of revolution whose perimeter was that obtained from the threshold contoured image. Digital image processing of clusters of over 100 alveolar air sacs gave an average alveolus interior volume of $(10.4 \pm 0.2) \times 10^4\ \mu m^3$.

Using the airway casting procedure followed by the vascular casting procedure, it was possible to obtain alveolar air sac and vascular casts simultaneously as shown in Figure 3. Here the cast had been pulled apart to reveal an alveolar air sac partially surrounded by the vasculature which remained after breakage. We observed that the simultaneous casting of air sacs and vasculature was not successful if the vascular casting preceded the airway casting. In explanation of this phenomenon, we suggest that if the vasculature were filled with polymer, any fluid which became trapped during subsequent polymer injection in the airways would drain more slowly than if the vasculature were clear and unobstructed.

Figure 4 shows a fractured vessel cross section in the bulk frozen tissue. The vessel is filled with blood and has many red blood cells oriented somewhat randomly in the fracture plane. On the digitized micrograph, thirty measurements of the maximum red blood cell dimension gave a red blood cell diameter of $7.2 \pm 0.4\ \mu m$ which is consistent with the commonly accepted rat red blood cell diameter measured by light optical techniques. Therefore, red blood cell dimensions can be used as an internal size calibration.

An alveolus intersected by a fracture plane is shown in Figure 5 where at least two alveolar capillaries containing red blood cells are seen in cross section along the right edge of the alveolus. From several digitized low magnification (about 200X) micrographs of frozen hydrated lung, we measured an average alveolus radius of $28 \pm 0.6\ \mu m$, and if we assumed spherical shape, this gave an alveolus volume of $(9.2 \pm 0.2) \times 10^4\ \mu m^3$, somewhat less than the value obtained from airway casts.

Figure 6 is an enlarged view of one of the alveolar capillary cross sections from Figure 5. From Figures 5 and 6 we can deduce that the alveolar capillaries lie fairly flat within the wall of the alveolus, and it appears that red blood cells squeeze through these flattened capillaries such that their surface area tangent to the alveolar wall would be optimal for gas exchange. From several digitized micrographs like Figure 5, we measured an average alveolar wall thickness of $2.5 \pm 0.8\ \mu m$ while the length to width ratio of an alveolar capillary cross section was consistently 4.8 ± 0.3.

Analysis and Discussion

The corrosion casting method is helpful in illuminating anatomical luminal features which are otherwise difficult to visualize in their three-dimensional context. It would seem that in addition to the descriptive interpretations made available by corrosion casting, it should be possible to make measurements providing that structural distortions due to the preparation method are well understood or that appropriate calibration procedures are utilized. A critical review of the corrosion casting technique is provided by Lametschwandtner et al. (1984).

Even though image processing of SEM micrographs of casts produced good measurements of cast areas and volumes, these numbers probably do not reflect the true dimensions of structures in the living lung. We felt that bulk frozen hydrated lung tissue would be dimensionally more accurate, and therefore, it was worthwhile to compare the casted and frozen preparations. Since our analysis of bulk frozen tissue was performed at low magnification incapable of showing structural alterations in the specimen which may have been caused by ice crystal damage from the freezing process (Echlin, 1978), it was assumed that the bulk frozen hydrated tissue preparation could be used to calibrate measurements of casts. This assumption was justified by measuring red blood cells of known dimensions. Other calibration methods could have been used, such as histological comparison (Nelson et al., 1984), but we wanted to retain the three-dimensional SEM view which could be compared directly with cast specimens.

By comparing Figure 5 with Figure 1, we observe that the alveolar capillaries revealed by the casting method do not appear to be flat. This suggests that one possible artifact caused by the casting of lung vessels is swelling. We further observe that the alveolus volume measured in the frozen tissue is approximately 10% smaller than that measured in the airway casts suggesting that polymer injection in the casting procedure may cause air sacs to enlarge under pressure. However, the three-dimensional connectedness of alveolar capillaries is preserved and appears to show interdigitation as reported by Guntheroth et al., (1982).

Alveoli may contain wall perforations which allow airspace connections with other surrounding alveoli as can be seen in Figures 5 and 1, but it is possible that some perforation damage arises during preparation. The approximate interior surface of an alveolus could be measured from Figure 5 by subtracting the total area occupied by perforations from the total alveolus surface area. But this measurement is not equal to the vascular surface area in the alveolus, since it can be seen, for example, that two alveolar capillaries in Figure 5 are not separated by a perforation but rather are connected by a thin portion of wall tissue. If one were to estimate vascular surface area from Figure 5, it probably would be an overestimate.

The cast in Figure 1 shows only the alveolar capillaries with all tissue removed, and therefore, is a better preparation for estimating vascular surface area. If we could measure the alveolar surface area, then we could compute the alveolar vascular surface

area. But micrographs like Figure 1 show that it would be quite difficult to measure alveolar surface area due to the irregular alveolus shape. A more fruitful approach to measuring alveolar surface area would involve a measurement of average alveolar volume and shape in a large population from which an estimate of surface area can be calculated. Figure 2 shows a lung airway cast where clusters of alveolar air sacs are apparent. We assumed that the single air sac volume as seen in the airway cast in Figure 2 was nearly equal to the interior alveolar volume of the vascular cast (Dilly, 1984), in Figure 1, and since Figure 3 shows that the alveolar airway and surrounding vasculature are in close contact, this assumption seems justified.

If we calibrate the vascular casts against air sac volumes adjusted to compare with frozen tissue alveoli, we calculated an alveolar solid surface area of $(1.0 \pm 0.3) \times 10^4\ \mu m^2$ which when multiplied by the 0.86 vascular surface fraction gave $(8.6 \pm 0.4) \times 10^3\ \mu m^2$ as the vascular surface area of an alveolus. Dividing this number by the alveolar volume gave approximately $0.09\ \mu m^{-1}$ as the alveolar capillary surface area per unit alveolar volume. Interestingly, this number when multiplied by the respiratory tidal volume of the rat would give a rough estimate of the equivalent capillary surface area being utilized during breathing in the normal lung.

Conclusions

This work attempted to describe a procedure which gave repeatable results in corrosion casting of rat lung airways. This procedure when coupled with vascular corrosion casting and bulk frozen hydrated tissue preparation was shown to facilitate a semi-quantitative assessment of lung structure. With digital image processing of micrographs, it was possible to make large numbers of measurements which would otherwise be difficult to obtain.

Even though artifactual shrinkage and distortion of specimen casts are not well understood, it may be possible to use corrosion casting judiciously to obtain measurements of physiologically relevant parameters. Accordingly, we have shown with lung tissue that the corrosion casting technique has applications beyond simple three-dimensional structural descriptions.

Acknowledgements

The author wishes to extend a special thanks to Dr. Arati Shah-Yukich for her expert handling of the corrosion casting technique and of the SEM. The W.M. Keck Foundation has generously supported the efforts of the author while specific funding of the project was provided by the Whitaker Foundation.

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Discussion with Reviewers

Reviewer I: How many capillaries were measured to obtain the stated value of 2.5 μm for the average alveolar wall thickness? Does this value pertain only to the thin portion of the alveolar-capillary membrane? The value of 2.5 μm is larger than that measured in morphometric studies of chemically fixed lungs. How can these different results be explained?

Author: The wall thickness measurement was obtained by averaging sections of wall from ten alveoli and includes regions of capillary vascularization as well as non-vascular wall. The non-vascular wall alone was closer to 1.7 μm in thickness. Differences between our measurements and those from chemically fixed lungs are probably due to differences in the locations of measured regions within each alveolus.

Figure 1: Vascular cast of rat lung showing several alveolar structures having an interdigitated arrangement of capillaries. Bar = 20 μm.

Figure 2: Airway cast of rat lung showing broncheolus from which alveolar air sacs are clustered. The air sacs are roughly ellipsoid in shape. Bar = 100 μm.

Figure 3: Airway and vascular cast of rat lung showing one alveolar air sac in close contact with surrounding capillaries. Bar = 20 μm.

Figure 4: Frozen hydrated rat lung tissue showing cross section of circulatory vessel filled with red blood cells at time of tissue freezing. As a calibration for size, the red blood cells are about 7.2 μm in diameter. Bar = 10 μm.

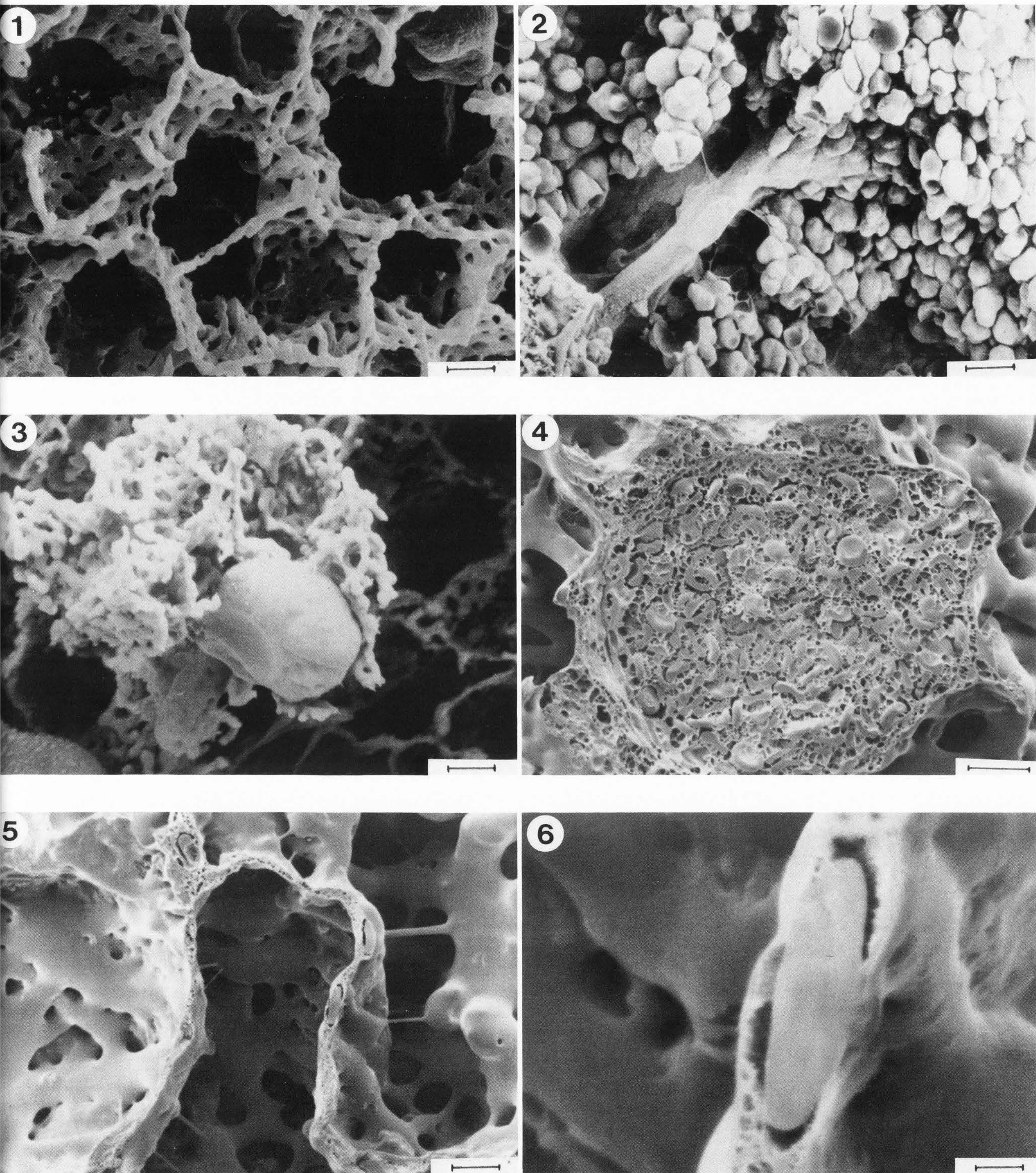

Figure 5: Frozen hydrated rat lung alveolus in cross section showing opening and walls of an alveolus. One of the alveolar capillaries is shown in figure 6. Bar = 10 μm.

Figure 6: Frozen hydrated rat lung alveolar capillary in cross section with single red blood cell passing through vessel. The average alveolar wall thickness is on the order of 2.5 μm and the red blood cell passes flat through and in intimate contact with the alveolar capillary. Bar = 1 μm.

Reviewer I: Figure 5 shows a high number of openings in the alveolar walls yet the typical alveolar surface should be nearly closed except for pores of Kohn. How is this explained? Also, what are the bridging strands seen in Figure 5?
Author: The bulk frozen lung preparation does show substantial perforation of the alveolar wall, and we do not have an explanation for this phenomenon if it is artifactual. The bridging strands were fluid at the time of freezing and could contain mucosal components which might have stretched between contacting wet surfaces during preparation.

Reviewer I: The lung by the nature of its function, is a very compliant organ where surface areas and volumes of alveoli and capillaries are critically dependent upon airway and vascular pressures. Can the author expect to calculate meaningful values of areas and volumes when the pressures were not controlled during the process of fixation, casting or freezing?
Author: We pointed out that measurements directly from casts were probably incorrect but that it was possible to calibrate the measurements and present them as semi-quantitative ratios which did relate to dimensional characteristics of the living tissue. Once the tissue is fixed, it is no longer compliant enough to warrant the same level of pressure control as with unfixed tissue.

A. Lametschwandtner: Do you agree that more accurate relationships between blood vessels and air spaces might be observed by fracturing a frozen cast of lung tissue which has been casted via the airways and the vasculature?
Author: Yes. We appreciate the suggestion and will probably try this at our next opportunity.

H. Ditrich: Could the dehydration of casts in graded ethanol result in softening or distortion of the casts, and is this step essential for good drying of the casts?
Author: It is possible that ethanol may cause distortion of the casts. We found that without using graded ethanol dehydration, our cast remained wet for excessively long periods of time and could not be pumped to operating vacuum in the SEM.