Four-dimensional visualization of subpleural alveolar dynamics in vivo during uninterrupted mechanical ventilation of living swine

Eman Namati,1,6 William C. Warger II,1,6 Carolin I. Unglert,1,2 Jocelyn E. Eckert,1 Jeroen Hostens,5 Brett E. Bouma,1,3 and Guillermo J. Tearney1,3,4,*

1Harvard Medical School and Massachusetts General Hospital, Wellman Center for Photomedicine, 40 Blossom St., BAR-714, Boston, MA 02114 USA
2Air Liquide Centre de Recherche Claude-Delorme, Medical Gases Group, 1 Chemin de la Porte des Loges, Les-Loges-en-Josas, France
3Harvard-MIT Division of Health Sciences and Technology, 77 Massachusetts Avenue, Cambridge, MA 02139 USA
4Department of Pathology, Massachusetts General Hospital, Boston, MA 02114 USA
5Bruker Corporation, Kontich, Belgium
6Co-first authors. These authors contributed equally to this work

*gtearney@partners.org

Abstract: Pulmonary alveoli have been studied for many years, yet no unifying hypothesis exists for their dynamic mechanics during respiration due to their miniature size (100-300 μm diameter in humans) and constant motion, which prevent standard imaging techniques from visualizing four-dimensional dynamics of individual alveoli in vivo. Here we report a new platform to image the first layer of air-filled subpleural alveoli through the use of a lightweight optical frequency domain imaging (OFDI) probe that can be placed upon the pleura to move with the lung over the complete range of respiratory motion. This device enables in-vivo acquisition of four-dimensional microscopic images of alveolar airspaces (alveoli and ducts), within the same field of view, during continuous ventilation without restricting the motion or modifying the structure of the alveoli. Results from an exploratory study including three live swine suggest that subpleural alveolar air spaces are best fit with a uniform expansion ($r^2 = 0.98$) over a recruitment model ($r^2 = 0.72$). Simultaneously, however, the percentage change in volume shows heterogeneous alveolar expansion within just a 1 mm x 1 mm field of view. These results signify the importance of four-dimensional imaging tools, such as the device presented here. Quantification of the dynamic response of the lung during ventilation may help create more accurate modeling techniques and move toward a more complete understanding of alveolar mechanics.

©2013 Optical Society of America

OCIS codes: (110.4500) Optical coherence tomography; (170.2655) Functional monitoring and imaging.

References and links

1. E. Roan and C. M. Waters, “What do we know about mechanical strain in lung alveoli?” Am. J. Physiol. Lung Cell. Mol. Physiol. 301(5), L625–L635 (2011).
2. M. Cereda, K. Emami, S. Kadlec, Y. Xin, P. Mongkolwisetwara, H. Profka, A. Barulic, S. Pickup, S. Månsson, P. Wollmer, M. Ishii, C. S. Deutschman, and R. R. Rizi, “Quantitative imaging of alveolar recruitment with hyperpolarized gas MRI during mechanical ventilation,” J. Appl. Physiol. 110(2), 499–511 (2011).
3. A. J. Hajari, D. A. Yablonskiy, A. L. Sukstanskii, J. D. Quirk, M. S. Conradi, and J. C. Woods, “Morphometric changes in the human pulmonary acinus during inflation,” J. Appl. Physiol. 112(6), 937–943 (2012).
4. D. A. Yablonskiy, A. L. Sukstanskii, J. C. Woods, D. S. Gierada, J. D. Quirk, J. C. Hogg, J. D. Cooper, and M. S. Conradi, “Quantification of lung microstructure with hyperpolarized 3He diffusion MRI,” J. Appl. Physiol. 107(4), 1258–1265 (2009).
5. J. Bickenbach, R. Dombinski, M. Czaplik, S. Meissner, A. Tabuchi, M. Mertens, L. Knels, W. Schroeder, P. Pelosi, E. Koch, W. M. Kuebler, R. Rossaint, and R. Kühlen, “Comparison of two in vivo microscopy techniques for visualizing alveolar mechanics,” J. Clin. Monit. Comput. 23(S), 323–332 (2009).

6. D. E. Carney, C. E. Bredenberg, H. J. Schiller, A. L. Picone, U. E. McCann II, L. A. Gatto, G. Bailey, M. Fillinger, and G. F. Nieman, “The mechanism of lung volume change during mechanical ventilation,” Am. J. Respir. Crit. Care Med. 160(5), 1697–1702 (1999).

7. B. D. T. Daly, G. E. Parks, C. H. Edmonds, C. W. Hibbs, and J. C. Norman, “Dynamic alveolar mechanics as studied by videomicroscopy,” Respir. Physiol. 24(2), 217–232 (1975).

8. J. D. DiRocco, L. A. Pavone, D. E. Carney, C. J. Lutz, L. A. Gatto, S. K. Landas, and G. F. Nieman, “Dynamic alveolar mechanics in four models of lung injury,” Intensive Care Med. 32(1), 140–148 (2006).

9. M. R. Looney, E. E. Thornton, D. Sen, W. J. Lamm, R. W. Glenny, and M. F. Krummel, “Stabilized imaging of immune surveillance in the mouse lung,” Nat. Methods 8(1), 91–96 (2011).

10. S. Meissner, L. Knels, C. Schnabel, T. Koch, and E. Koch, “Three-dimensional Fourier domain optical coherence tomography in vivo imaging of alveolar tissue in the intact thorax using the parietal pleura as a window,” J. Biomed. Opt. 15(1), 016030 (2010).

11. S. Meissner, A. Tabuchi, M. Mertens, W. M. Kuebler, and E. Koch, “Virtual four-dimensional imaging of lung parenchyma by optical coherence tomography in mice,” J. Biomed. Opt. 15(3), 036016 (2010).

12. M. Mertens, A. Tabuchi, S. Meissner, A. Krueger, K. Schirrmann, U. Kertzscher, A. R. Pries, A. S. Slutsky, E. Koch, and W. M. Kuebler, “Alveolar dynamics in acute lung injury: heterogeneous distortion rather than cyclic opening and collapse,” Crit. Care Med. 37(9), 2604–2611 (2009).

13. A. P. Moreci and J. C. Norman, “Measurements of alveolar sac diameters by incident-light photomicrography. Effects of positive-pressure respiration,” Ann. Thorac. Surg. 15(2), 179–186 (1973).

14. G. F. Nieman, C. E. Bredenberg, W. R. Clark, and N. R. West, “Alveolar function following surfactant deactivation,” J. Appl. Physiol. 51(4), 895–904 (1981).

15. H. J. Schiller, U. G. McCann 2nd, D. E. Carney, L. A. Gatto, J. M. Steinberg, and G. F. Nieman, “Altered alveolar mechanics in the acutely injured lung,” Crit. Care Med. 29(5), 1049–1055 (2001).

16. D. Schwenninger, H. Runck, S. Schumann, J. Haberstroh, S. Meissner, E. Koch, and J. Guttmann, “Intravital microscopy of subpleural alveoli via transthoracic endoscopy,” J. Biomed. Opt. 16(4), 046002 (2011).

17. J. Steinberg, H. J. Schiller, J. M. Halter, L. A. Gatto, M. Dasilva, M. Amato, U. G. McCann, and G. F. Nieman, “Tidal volume increases do not affect alveolar mechanics in normal lung but cause alveolar overdistension and exacerbate alveolar instability after surfactant deactivation,” Crit. Care Med. 30(12), 2675–2683 (2002).

18. J. M. Steinberg, H. J. Schiller, J. M. Halter, L. A. Gatto, H.-M. Lee, L. A. Pavone, and G. F. Nieman, “Alveolar instability causes early ventilator-induced lung injury independent of neutrophils,” Am. J. Respir. Crit. Care Med. 169(1), 57–63 (2004).

19. W. W. Wagner, Jr., “Pulmonary microcirculatory observations in vivo under physiological conditions,” J. Appl. Physiol. 26(3), 375–377 (1969).

20. H. Liu, H. Runck, M. Schneider, X. Tong, and C. A. Stahl, “Morphometry of subpleural alveoli may be greatly biased by local pressure changes induced by the microscopic device,” Respir. Physiol. Neurobiol. 178(2), 283–289 (2011).

21. Y. Wu and C. E. Perlman, “In situ methods for assessing alveolar mechanics,” J. Appl. Physiol. 112(3), 519–526 (2012).

22. D. Huang, E. A. Swanson, C. P. Lin, J. S. Schuman, W. G. Stinson, W. Chang, M. R. Hee, T. Flotte, K. Gregory, C. A. Puliafito, and J. G. Fujimoto, “Optical coherence tomography,” Science 254(5035), 1178–1181 (1991).

23. N. Hanna, D. Saltzman, D. Mukai, Z. Chen, S. Sasse, J. Milliken, S. Guo, W. Jung, H. Colt, and M. Brenner, “Two-dimensional and 3-dimensional optical coherence tomographic imaging of the airway, lung, and pleura,” J. Thorac. Cardiovasc. Surg. 129(3), 615–622 (2005).

24. L. Kirsten, M. Gaertner, C. Schnabel, S. Meissner, and E. Koch, “Four-dimensional imaging of murine subpleural alveoli using high-speed optical coherence tomography,” J. Biophotonics 6(2), 148–152 (2013).

25. C. I. Unglert, E. Namati, W. C. Warger 2nd, L. Liu, H. Yoo, D. K. Kang, B. E. Bouma, and G. J. Tearney, “Evaluation of optical reflectance techniques for imaging of alveolar structure,” J. Biomed. Opt. 17(7), 071303 (2012).

26. S. H. Yun, G. J. Tearney, J. F. de Boer, N. Ifrimia, and B. E. Bouma, “High-speed optical frequency-domain imaging,” Opt. Express 11(22), 2953–2963 (2003).

27. S. H. Yun, G. J. Tearney, B. J. Vakoc, M. Shishkov, W. Y. Oh, A. E. Desjardins, M. J. Suter, R. C. Chan, J. A. Evans, I.-K. Jang, N. S. Nishioka, J. F. de Boer, and B. E. Bouma, “Comprehensive volumetric optical microscopy in vivo,” Nat. Med. 12(12), 1429–1433 (2007).

28. W. S. Rashband and J. Image, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/. 1997–2011.

29. R. P. Woods, S. R. Cherry, and J. C. Mazzotti, “Rapid automated algorithm for aligning and reslicing PET images,” J. Comput. Assist. Tomogr. 16(4), 620–633 (1992).

30. E. Namati, J. De Ryk, J. Thiesse, Z. Towfic, E. Hoffman, and G. Mclennan, “Large image microscope array for the compilation of multimodality whole organ image databases,” Anat. Rec. (Hoboken) 290(11), 1377–1387 (2007).

31. J. Gil, H. Bachofen, P. Gehr, and E. R. Weibel, “Alveolar volume-surface area relation in air- and saline-filled lungs fixed by vascular perfusion,” J. Appl. Physiol. 47(5), 990–1001 (1979).
1. Introduction

Pulmonary alveoli are air-filled sacs that terminate the distal airways within the normal lung. Together with a dense capillary network, the alveoli provide the structural means for oxygen and carbon dioxide exchange during the respiratory cycle. While gas exchange is crucial to sustaining life, no unifying hypothesis exists for the dynamic mechanics of alveoli during respiration [1]. In particular, it is unclear whether alveoli expand isotropically as a single unit (uniform expansion), heterogeneously as separate compartments (recruitment), or by a combination of mechanisms. The lack of consensus derives from the size of alveoli (100-300 μm diameter in humans) and the constant motion during respiration that prevents conventional three-dimensional imaging techniques from visualizing continuous dynamics of individual alveoli in vivo [2,3]. This limitation currently requires non-invasive three-dimensional imaging techniques to assume an approximate number, size, and state (open or closed) of alveoli within a given region based on geometric modeling [4] alone. Crucial information has been obtained with the use of suction and/or a coverslip pressed against the pleura to maintain the field of view in vivo [5–19], but questions have been raised about how these configurations affect the natural motion and structure of the lung [20,21]. Here we describe a lightweight optical-imaging probe that can be placed upon the pleura during an open-chest procedure and moves with the ventilated lung. This device, therefore, maintains the field of alveoli without restricting motion, and enables the acquisition of four-dimensional microscopic images over multiple respiratory cycles during continuous ventilation in vivo.

Optical coherence tomography (OCT) [22] is an optical imaging technique that has shown promise for providing three-dimensional microscopic-resolution snap-shots of alveoli through the pleura [10–12,16] and bronchioles [23] in-vivo, and four-dimensional images of an alveolar cluster in situ [24]. Optical frequency domain imaging (OFDI), a second-generation OCT technique, provides significantly increased acquisition rates for volumetric imaging
within 1-2 mm of the surface in isotropic tissue [25–27]. Within this paper, we report the development of a miniature OFDI scanning probe that can be placed upon the pleura to acquire three-dimensional images of subpleural alveolar dynamics during continuous ventilation in vivo. We also demonstrate the ability of OFDI to visualize four-dimensional alveolar dynamics within living swine to provide insights on subpleural alveolar expansion.

2. Methods

2.1 Surgical procedure

Male Yorkshire swine (n = 3) weighing 30-35 kg were initially sedated with a mixture of Telazol 4.4 mg/kg and Xylazine 2.2 mg/kg, intubated with a cuffed endotracheal tube, and placed on mechanical ventilation with anesthesia maintained using 1-3% isoflurane. Buprenorphine 0.3 mg/kg was also administered intravenously for additional pain management. ECG, blood pressure, pulse oximetry, and temperature were monitored for assessment of anesthesia, pain, and distress. Swine were initially ventilated at 20 bpm and 20 cmH₂O peak inspiratory pressure (PIP). Once a surgical plane of anesthesia had been met, the swine were positioned on their left side and a right thoracotomy was performed to reveal the right middle lobe. Warm saline was applied to the pleural surface to ensure the tissue retained moisture. The lightweight probe was placed at various locations on the right middle lobe in Fig. 1, and continuous volumetric data sets were acquired over a minimum of three breaths at various ventilation settings (10 bpm, 1-3 cmH₂O positive end-expiratory pressure (PEEP), 11-20 cmH₂O PIP). Pressure and flow sensors positioned at the proximal tip of the endotracheal tube recorded the intratracheal ventilation parameters during image acquisition. Once the imaging procedure was completed, the animal was euthanized via exsanguination, the endotracheal tube was clamped to keep the lungs inflated, and the lungs were carefully excised. The protocol was approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Fig. 1. Picture of the MEMS probe with a diagram of the primary components within the MEMS probe and the positioning on the right middle lobe of live swine on mechanical ventilation. An optical fiber and electrical cable connect the probe to the OFDI system.
2.2 OFDI imaging of 4D alveolar dynamics

The OFDI system used in this study images a 256x256x1024 pixel volume in 1.05 s (62.5 kHz swept-source laser) with an imaging depth of 1-2 mm in isotropic tissue. Because individual alveoli are relatively small with thin walls, it is crucial to create a high-resolution imaging device, which results in a depth of field less than the total translation of an open lung during a respiratory cycle. To overcome this limitation, we developed a lightweight (12 g) cylindrical (23 mm diameter, 23 mm tall) probe with a combination of a 0.2 NA imaging lens and a dual-axis microelectromechanical systems (MEMS) mirror (Mirrorcle Technologies, Inc. Richmond, CA) that scans a 930 µm x 930 µm transverse field of view in Fig. 1. The lightweight probe can then be placed on top of the pleura without suction or attachment techniques to continuously acquire images over the respiratory cycle. A single highly-flexible fiber-optic and an electrical cable connect the device to the imaging console in Fig. 1. An open space exists between the imaging lens and the field of view such that nothing is in direct contact with the imaged region.

However, it is important to note that the 12 g weight and 415 mm² cross-sectional area of the probe in contact with the lung provides an inherent pressure to the pleura immediately surrounding the field of view, which we have calculated to be 3.0 cmH₂O.

It is important to note, that while the method presented here is currently limited to positive-pressure ventilation, the optical probe could be fixed between the ribs and a negative seal re-established between the lung and chest wall to investigate a negative-pressure model. However, this configuration requires alternative techniques to maintain the field of view across the respiratory cycle.

2.3 Image processing, registration, and analysis

Each resultant OFDI volume was cropped to 256x256x256 pixels to reduce the file size and computation time, and then filtered with a fast Fourier-transform bandpass filter in ImageJ [28] to suppress vertical stripes that resulted from strong back reflections from the pleural surface. To reduce slight motion between volumes (<150 µm) each complete time-series was registered using a three-dimensional registration open-source algorithm (Automated Image Registration (AIR) [29]). Upon completion of the processing, the pixel sizes were adjusted in ImageJ to provide an isotropic 3.6 µm pixel size.

A single data set at PIP was assessed to label each alveolar air space within the field of view. An air space was defined as a region under the pleura with contrast similar to the air region above the pleura and was closed in all transverse directions to ensure the complete alveolar air space could be segmented. The boundary of each alveolar air space was chosen as the interpretation of the inner alveolar wall and traced manually over 18 volume acquisitions (6 samples per breath). The analysis was limited to the first layer of subpleural alveoli due to imaging artifacts that result from the propagation of light through tissue-air interfaces. A boundary was manually created along the bottom of the air space to close any ducts or pathways to additional air spaces in the axial direction by connecting the natural incomplete curves of the alveolar walls where the bottom of the alveolar air space narrows. It is important to note that we did not manually create boundaries between alveoli in the transverse direction, such that each alveolar air space may be comprised of multiple (1-5) alveoli, thereby limiting our analysis to clusters of alveoli and ducts and not individual alveoli.

Upon completion of the segmentation, the individual segments were stacked together to create three-dimensional volumes. A dilation and erosion algorithm was applied in the direction orthogonal to the segmentation plane in Matlab (Mathworks: Natick, MA) to smooth any slight mismatch between segments in adjacent frames. Four-dimensional renderings were created with OsiriX (Pixmeo SARL: Bernex, Switzerland). Volume and surface area were calculated for each alveolar air space by summing the number of voxels within the three-dimensional shape and along the surface, respectively, and then multiplying
by the appropriate pixel size. Total alveolar volume ($V_T$) and total alveolar surface area ($SA_T$) was calculated by summing the volume and surface area of all alveolar air spaces during a single volume acquisition (time point), respectively. Percentage change in volume ($\Delta V\%$) and surface area ($\Delta SA\%$) was defined as the percent change of alveolar volume and surface area for each volume acquisition with respect to the minimum of the subset.

### 2.4 OFDI alveolar imaging validation

A comparison of alveolar air spaces segmented from OFDI images were compared with the same air spaces segmented from micro-CT images to confirm the volumes and surface areas measured from OFDI images are representative of the true volume and surface area. A freshly excised swine lung from a separate male Yorkshire swine was instillation fixed with a gravity-feed system consisting of 10% formaldehyde solution, 10% ethanol, 25% polyethylene glycol 400, and 55% laboratory distilled water, and then dried at 20 cmH$_2$O [30]. A 5 mm diameter, 5 mm tall cylinder was cut from the right middle lobe, where the top circular surface of the cylinder contained the pleura. Images were acquired with a high-resolution micro-CT system (SkyScan 1172, Kontich, Belgium) at atmospheric pressure containing an isotropic 2.9 µm$^3$ voxel size. Each sample was rotated approximately 196$^\circ$ at 0.4$^\circ$ steps with 4 projection images averaged per rotation step, and no external contrast was applied. Images of the subpleural alveoli within the same field of view were also acquired with the OFDI system described above and a bench-top (non-contact) galvanometer-based scanning system. A bicubic interpolation was applied to the OFDI images in ImageJ to match the isotropic 2.9 µm$^3$ pixel size between data sets, and then both data sets were rotated in three-dimensions to match axial cross sections. Eighteen identical alveolar air spaces from the right middle lobe sample were segmented in the OFDI data set and then the micro-CT data set by a single user, as described in the Image Processing, Registration, and Analysis Section, with approximately 3 days between data sets to prevent bias.

### 2.5 Contact probe imaging validation

We conducted an additional set of experiments to confirm that the 3 cmH$_2$O pressure applied to the pleura surrounding the field of view, induced by the weight of the device (12.13 g) exerted over the cross-sectional area of the device base (4.15 cm$^2$), did not significantly affect the segmented volumes. A set of freshly excised lungs (<1 hr postmortem) from a fifth male Yorkshire swine was ventilated upon the bench-top with 5 cmH$_2$O PEEP and 20 cmH$_2$O PIP for approximately 5 minutes to recruit any areas of atelectasis, and then maintained at 20 cmH$_2$O constant pressure. A piece of paper with a 2 mm diameter center hole was placed on top of the pleura and wetted with saline to preserve the field of view and prevent the tissue from drying. An image was acquired with the non-contact scanning system at constant pressures of 20, 15, 10, and 5 cmH$_2$O while optimizing the position of the bench-top scanner at each pressure to ensure the image remained in-focus. The lung was then re-inflated to 20 cmH$_2$O, wetted with saline, and data sets were acquired with the probe placed on top of the lung for the same pressure steps. Seventeen matching alveolar air spaces within the data sets were segmented at each of the pressure steps by two blinded observers with no prior knowledge of the segments created by the other observer.

### 2.6 Modeling and statistical analyses

Group data was non-normally distributed according to the Shapiro-Wilk normality test performed with Prism (GraphPad; La Jolla, CA), and reported as median (25th, 75th percentiles). Correlations were analyzed with the Spearman rank test and unpaired groups were compared with a Mann-Whitney test. A $P$ value $\leq$0.05 was considered significant.

Our measurement of regional alveolar dynamics was calculated by a linear regression of $V_T$ versus $SA_T$ for each imaged volume on logarithmic scale: $\log(SA_T) = \beta_1(\log V_T) + \alpha$, where $\beta_1$ is the regression coefficient and $\alpha$ is the y-intercept calculated with Prism. The coefficient...
of determination ($r^2$) and the 95% confidence interval of the regression coefficient were recorded. A non-linear regression was also calculated for both models, where $\beta_1 = 2/3$ (uniform expansion) simulates uniform spherical expansion from the mathematical representation of a sphere: $V_T = SAT^{2/3}$ over the imaged time points, and $\beta_1 = 1$ (recruitment) simulates a prolonged increase in volume compared to the surface area ($V_T = SAT$) as the recruited alveoli add extra volume with minimal change in surface area [31].

The individual alveolar air spaces were also analyzed in a purely statistical manner to provide some insight on whether alveoli expand by uniform expansion, septal folding, or a combination of expansion and folding. To a first approximation, we assume a similar mechanism to recruitment where septal pleats would separate heterogeneously due to confounding effects such as neighboring alveoli, surfactant, and mucus, resulting in a prolonged increase in alveolar volume compared to a slow continuous increase in surface area: $SA = \beta_1 V$. This compares to uniform expansion where we assume the spherical relation holds over the imaged time points and the numeric change in volume slows compared to the surface area: $SA = \beta_3 V^{2/3}$. We then applied a multiple regression to each alveolar air space with SAS (SAS Institute Inc.: Cary, NC) and a combination of expansion and folding was recorded when a positive contribution of both models ($\beta_2>0, \beta_3>0$) significantly improved the fit of each model alone. When a positive combination of models did not significantly improve both individual models, the best-fit model was recorded based on the smallest root-mean-squared error.

3. Results

3.1 OFDI validation

Figure 2(A)-2(B) shows representative axial cross sections from the OFDI and micro-CT data sets with five matched alveolar air spaces. From Fig. 2(C)-2(D) we observe that OFDI underestimates volume and surface area compared to micro-CT (slope of 1.38 and 1.15 respectively). This was not surprising because OFDI overestimates wall thickness due to refraction effects from the light traversing large refractive index changes between the lung parenchyma and air-filled alveoli [32]. Simultaneously, we do observe high correlation between the segmented volumes ($r = 0.96, \ P < 0.0001$) and surface areas ($r = 0.98, \ P < 0.0001$) for the 18 matched alveolar air spaces within the first layer of the OFDI and micro-CT data sets. Therefore we can conclude that absolute volume and surface area calculations are underestimated but the relative change can be accurately measured using OFDI.

![Fig. 2. (A) OFDI and (B) micro-CT axial cross sections of fixed subpleural swine alveoli. High correlation of segmented (C) volume ($V_{micro-CT} = 1.38V_{OFDI} + 0.003$) and (D) surface area ($SA_{micro-CT} = 1.15SA_{OFDI} + 0.0006$) show relative measurements of subpleural alveolar dynamics can be measured accurately with OFDI. Scale bar = 200 µm.]

3.2 Contact-probe validation

Figure 3(A)-3(B) show matched axial cross sections between the non-contact bench-top and contact MEMS-probe data sets. Figure 3(C) shows high correlation ($r = 0.88, \ P < 0.0001$) for
17 matched alveolar air spaces between the contact and non-contact segments, leading to the assumption that the contact probe does not create a significant affect on the measurement of alveolar air spaces. However, because the MEMS probe provides a higher lateral resolution 4.0 µm (in air) versus 10.0 µm (in air) for the bench-top system, we observed that the bench-top system significantly underestimates alveolar volume compared to the probe (slope of 1.48).

### 3.3 Four-dimensional visualization of alveolar dynamics

Following demonstration that the placement of the lightweight probe upon the pleura does not affect the measurement of relative changes in alveolar volume and surface area, and the relative measurements are accurate from a comparison of OFDI and micro-CT images of fixed swine lung, we confirmed the utility of our device for imaging alveoli in vivo within male Yorkshire swine in the left lateral recumbent position. Figure 4 provides a representative four-dimensional visualization for one respiratory cycle consisting of 34 subpleural alveolar air-spaces ventilated at 10 bpm, 3 cmH2O PEEP, and 20 cmH2O PIP. The first and second columns demonstrate en-face and axial cross-sectional planes from the OFDI acquisition, respectively. The third column shows the expansion and contraction of the segmented alveolar air spaces in three dimensions during the breath. The fourth column provides a maximum intensity projection (MIP) of the en-face plane where the color represents ΔV% from the minimum volume of each air space during the respiratory cycle. The fifth column shows the pressure profile over the respiratory cycle, where the asterisk (*) represents the mean pressure during the volume acquisition. A complete movie of the 4D visualization over three respiratory cycles is provided in Fig. 5 (Media 1).

It is important to note the heterogeneity of the alveolar expansion visualized within the MIP of the en-face plane in 4D, where some alveolar air spaces expand and contract with the pressure profile as expected, and others maintain minimal volume change within just a 1 mm x 1 mm field of view. The heterogeneity of alveolar expansion can be further visualized in Fig. 6. The histogram of the maximum ΔV% for each alveolar air space in Fig. 6(A) shows a large range of maximum expansion, where the majority of alveolar air spaces expanded between 20 and 160% from their respective minimum volumes. The scatter plot in Fig. 6(B) shows the segmented volume for each alveolar air space vs. the intratracheal pressure at the time of acquisition for the air space, further supporting the observation of heterogeneous expansion.
Fig. 4. (A) En-face and (B) axial cross-sectional OFDI images acquired over one respiratory cycle in vivo with swine continuously ventilated at 10 bpm, 3 cmH2O PEEP, 20 cmH2O PIP. (C) Four-dimensional rendering of 34 segmented subpleural alveolar air spaces. (D) Maximum intensity projection (MIP) en-face visualization of segmented alveoli color-coded to percentage volume change of each alveolar air space over the respiratory cycle. (E) Intratracheal pressure trace during the respiratory cycle where * denotes mean intratracheal pressure during the volume acquisition within 1.05 s. Scale bar = 100 μm.
Fig. 5. Representative four-dimensional visualization of 34 subpleural alveolar air spaces over three breaths in healthy swine ventilated at 10 bpm, 3 cmH₂O PEEP, and 20 cmH₂O PIP. OFDI images were acquired with a lightweight probe placed directly upon pleura during a thoracotomy procedure (Media 1). The top-left panel is an en-face view and the bottom-left panel is an axial cross-sectional view from the OFDI data sets. The top-center panel provides an en-face view and the bottom-center panel provides an isometric view of the segmented alveolar air spaces. The top-right panel is a maximum intensity projection of the segmented alveoli within the en-face plane where the color represents the percent change in volume of each alveolar air space with respect to the minimum volume during the three breaths. It is important to note the heterogeneity of the alveolar expansion where some alveoli change volume with the change in pressure and others undergo minimal change. The bottom-right panel provides the pressure profile over the 3 respiratory cycles, where the asterisk (*) represents the mean pressure during the volume acquisition.

Fig. 6. (A) Histogram of maximum ΔV% and (B) scatter plot of air space volume vs. intratracheal pressure during the acquisition show heterogeneous alveolar expansion within the 1 mm x 1 mm field of view of subpleural alveoli.

3.4 Alveolar dynamics

Figure 7(A) plots ΔV% with respect to the minimum VT in addition to the relative change in mean intratracheal pressure during the acquisition. The alveolar air-space volume increases...
immediately upon pressure increase during forced positive-pressure ventilation, and a clear
delay was observed for the volume to decrease as the pressure reduced to zero and the lung
was allowed to exhale naturally. The dynamic intratracheal pressure-volume (PV) curve in
Fig. 7(B) was calculated by integrating the continuous intratracheal flow, and compared to the
alveolar PV curve in Fig. 7(C), which was created by plotting $V_T$ versus the mean
intratracheal pressure during the volume acquisition. The PV-curve scatter plots were fit by
the equation: $V = a + b[1 - \exp(-(P - c)/d)]$ where $V$ is Volume, $P$ is pressure, and $a$, $b$, $c$, $d$ are
fit parameters [33]. It is important to note that the alveolar PV curve simulates the lung
compliance curve, which does not contain the asymptotic tail at the end of expiration in the
intratracheal PV curve, and is consistent with the removal of the chest wall within the imaged
field of view [34]. It is also important to emphasize that the PV curve was acquired during
continuous ventilation to more accurately model continuous respiration compared to
interrupted static models.

3.5 Mechanism of alveolar expansion/contraction

Figure 8(A) shows the mathematical relation of $V_T$ and $SA_T$ for all of the alveoli within each
volume acquisition on logarithmic scale. A linear regression provided the relationship:
$\log(SA_T) = (0.660 \pm 0.012)\log(V_T) + 0.027$, with a 95% confidence interval of 0.635–0.685 for
the slope. A non-linear regression was also applied to the data in Fig. 8(B) to confirm the
difference between the fits for pure uniform expansion ($r^2 = 0.98$) and recruitment models ($r^2
= 0.72$).

Fig. 8. Logarithmic plot of $SA_T$ versus $V_T$ of subpleural swine alveoli within a 930 $\mu$m x 930
$\mu$m field of view continuously ventilated in vivo. (A) The best fit line was defined: $\log(SA_T) = $(0.660 $\pm$ 0.012) $\log(V_T) + 0.027$. (B) The comparison of pure recruitment (dashed blue line) and
uniform expansion (solid red line) models reinforce that the subpleural alveolar dynamics were
best fit with a uniform expansion model.
Multiple regression of the individual alveolar air spaces over 3 breaths showed that 7 of the 91 air spaces (7.7%) were best fit with a combination of folding and uniform expansion based on the mathematical models described in the modeling and statistical analyses section. Of the remaining air spaces, 8 were best fit with a folding model (8.8%) and 76 were best fit with a uniform expansion model (83.5%). This analysis suggests that subpleural alveoli undergo uniform expansion, folding, and a combination of both, but the use of uniform expansion alone would provide an adequate first approximation to individual subpleural alveolar dynamics.

4. Discussion

We have shown that a lightweight OFDI probe can be positioned directly upon the pleural surface of a live swine to acquire four-dimensional images of subpleural alveolar dynamics during continuous ventilation. The segmentation of the first layer of air-filled subpleural alveolar air spaces provided a four-dimensional visualization of the alveolar volume change that could then be used to analyze alveolar dynamics. A comparison of micro-CT and OFDI images showed that the segmented volumes and surface areas from OFDI images of fixed lung parenchyma were highly correlated \( r = 0.96; P < 0.0001 \) for volume and \( r = 0.98; P < 0.0001 \) for surface area, however the wall thickness is overestimated in the OFDI images suggesting that the relative changes in volume and surface area, not absolute change, can be measured accurately. Utilizing previously established models of plotting \( V_T \) versus \( SA_T \) during each volume acquisition suggests that subpleural swine alveoli were best fit with uniform expansion \( (r^2 = 0.98) \) in comparison to recruitment \( (r^2 = 0.72) \) within a 930 \( \mu \)m x 930 \( \mu \)m region of the right middle lobe. The relationship between surface area and volume was determined to be \( \log(SA_T) = (0.660 \pm 0.012)\log(V_T) + 0.0272 \), which is in good agreement with previous analyses performed on fixed lung samples from rats [35], dogs, guinea pigs, and rabbits [36]. The analysis of 91 alveolar air spaces segmented across 18 time points (3 breaths) also suggests that both uniform expansion and folding effects contribute to alveolar dynamics, but the use of a pure uniform expansion model provides an accurate first approximation.

The uniform expansion model fit is consistent with the fact that we have yet to observe any incidences of recruitment in healthy swine lung. There is a possibility that de-recruited alveoli exist between the alveolar air spaces we segmented, but we retained a PIP \( \leq 20 \text{ cmH}_2\text{O} \) to image during normal physiologic respiratory parameters and prevent bleb formation. It is also important to reemphasize that this analysis was limited to the first layer of subpleural alveoli where the top surface was mechanically attached to the pleura, and as such, it is possible that the thicker pleura could prevent the subpleural alveoli from de-recruiting. Another interesting observation is that, while the uniform expansion model fits the data better statistically, there was obvious heterogeneous expansion. This observation seems to support the hypothesis by Gil and Weibel [37] that recruitment within healthy undamaged lungs comprises a major depreciation in alveolar size and does not involve complete alveolar collapse. The observation is also consistent with the hypothesis by Hajari et al. that alveoli within MRI diffusion images that are assumed to be closed could in fact have some nonzero trapped gas volume, which would lead to an overestimation of the total number of alveoli [3] and thereby an inaccurate representation of the role of recruitment within the lung. Additional studies are required with animal models that exhibit different attributes, such as collateral ventilation, in order to determine whether alveolar recruitment can be visualized in four dimensions during continuous ventilation of healthy, undamaged lungs. Also, the disparity between the models developed from \textit{ex-vivo} samples and our \textit{in-vivo} imaging results emphasizes the importance of 1) three-dimensional static \textit{in situ} or \textit{ex-vivo} imaging of fresh tissue using techniques such as micro-CT with well developed protocols and sample sizes across multiple pressures or 2) four-dimensional imaging during continuous respiration with
smaller sample sizes to create accurate conclusions about alveolar dynamics without the use of global mathematical and geometric assumptions.

When interpreting the PV-curve data, it is important to emphasize that these results are based on a dynamic pressure-volume model with continuous ventilation and not a static or quasi-static model where ventilation stops incrementally during respiration. While an argument can be made in support of dynamic models because they are more representative of normal breathing, the static models have provided the basis for ventilation strategies used to reduce ventilator-induced lung injuries [38,39]. One reason for the predominant use of static ventilation models is the accuracy of the pressure measurement as resistance within the lung caused by various diseased states can result in a falsely elevated pressure measurement [38,39]. While these results provide a small insight at dynamics within a healthy lung, additional studies involving the use of this device with a static model in both normal and diseased/injured states could provide the means to compare the physiologic changes between the two models at the alveolar level.

It is important to note that the heterogeneous expansion throughout the field of view could also correspond to differences in expansion between separate acini, bronchioles, and/or ducts. It is also difficult to discriminate between individual alveoli and alveolar ducts within the resultant images, yet the data is likely weighted more towards individual alveoli considering we are imaging sub-pleural alveoli where we expect a limited amount of any terminating ducts at the pleural wall. As such, the chosen segmentation conditions limited our analysis to alveolar air spaces, which incorporated one to five alveoli within the first layer below the pleura. This condition provided the most accurate representation of the alveolar wall provided by OFDI images by only utilizing artificial boundaries to restrict airways leading to regions where refraction artifacts prevent an accurate representation of the air-filled lung. However, we are currently unable to determine whether neighboring alveolar air spaces are interconnected below the first layer of alveoli, and how the alveolar dynamics we observed for alveolar airspaces connected to the pleura correspond to subsurface alveoli surrounded by other alveoli. Further experimentation, such as registered micro-CT images with the OFDI field of view and/or index-matching the alveolar air spaces [40,41], is required to identify the pathways and investigate the heterogeneous expansion dependencies for alveoli throughout the lung.

An analysis of images acquired with a non-contact bench-top imaging system and the MEMS-based imaging probe on intact ex-vivo swine lung supports our assumption that the contact probe does not affect the measurement of alveolar air spaces within our positive-pressure open-chest model. Further validation studies are required to assess the influence of the probe in a low-pressure model with pressures less than 5 cmH₂O. A more important influence that prevented the measurement of alveolar dynamics within a complete natural environment was the removal of the chest wall from the imaged region. The fixed depth of field of optical techniques requires the device to not only be in close proximity to the region of interest, but also retain the separation distance when the movement is larger than the depth of field. This was the primary justification for the development of a lightweight contact-based imaging probe since a non-contact system would require complex dynamic tracking and motorized positioning for both the transverse and axial directions. An additional anatomic limitation was that the analysis was limited to subpleural alveolar dynamics of the right middle lobe within the left lateral recumbent position. It is known that the use of suction and attachment techniques may influence the natural motion of subpleural alveoli [1,20,21]. We attempted to remove as many of these confounding influences by simply positioning the probe on top of the pleura and allowing gravity to retain the position of the probe. In addition, unlike prior in-vivo optical-imaging studies, this approach provided the opportunity to assess alveoli from regions of the lung that exhibit large respiratory motion. However, it is important to note that our observations result from a limited field of view (less than 1 mm x 1 mm) that may not be directly representative of the entire lung or the natural lung motion of the lung.
within a closed chest. Further four-dimensional \textit{in-vivo} imaging studies should be undertaken with well-established post-mortem stereology techniques to provide direct insight into various regions imaged \textit{in vivo} versus whole lung alveolar size/number metrics. In addition, further studies with a negative-pressure model and/or a miniature probe could provide insight into alveolar dynamics within a more natural environment.

An important conclusion from the micro-CT/OFDI analysis was the limitation of analyzing the first layer of subpleural alveoli. While questions remain whether the first layer of alveoli on both the pleural and airway regions are representative of the alveoli in between, there is no technique currently available to image air-filled internal alveoli with adequate temporal and spatial resolution to resolve individual alveoli during continuous respiration without puncturing the parenchyma. The use of alveolar lavage to replace the air with index-matched fluid could increase the number of visible layers from the pleura \cite{41} and the first punctured layer of alveoli surrounding a needle \cite{40}, but additional studies are required to characterize the differences between air-filled and fluid-filled alveoli to extrapolate the results from lavage and liquid ventilation to the natural environment. This analysis also showed that the current OFDI reconstruction of the alveolar air spaces did not provide exact discrete volume and surface-area measurements compared to micro-CT. As the light propagates through tissue, it undergoes both bulk scattering within the tissue and refraction at refractive index mismatches. OCT techniques generally assume the light propagates in straight lines, but the large refractive index mismatch between the tissue and air within the lung parenchyma results in refraction. This effect could be minimized for studies where exact measurements are required by filling the alveoli with a solution that contains a refractive index closer to tissue \cite{40,41}, or by deriving a numerical correction for the measured shape from the OCT image \cite{32}. While bulk scattering provides the contrast mechanism within the tissue, it also distorts the focal spot and degrades the resolution. These factors limit our ability to trace the exact boundary of the alveolar air spaces, as there may be miniature folds within the septa that are smaller than the resolution of the system, as seen with electron microscopy \cite{35,42–45}. Additional studies are required to determine the primary mechanism for the reconstruction error and formulate correction techniques to provide more exact measurements of alveolar volume and surface area. However, it is important to reiterate that the relative change in volume and surface area measured by OFDI images correlates extremely well with the measures from micro-CT images, which allows us to assume these relative measurements are accurate.

6. Summary and conclusions

From this study we can make the following conclusions: 1) a miniature lightweight optical probe can be placed upon the pleura to acquire four-dimensional alveolar dynamics within the first layer of air-filled subpleural alveoli \textit{in-vivo}, 2) relative changes in alveolar volume and surface area can be accurately measured with OFDI, 3) the use of previously defined mathematical models applied to the data presented in this work indicated, regional subpleural alveolar dynamics of healthy undamaged swine lung were best fit with a uniform expansion model, and alveolar expansion was most accurately described by the combination of uniform expansion and septal folding, and finally 4) expansion of alveolar airspaces in a normal swine model was found to be highly heterogeneous emphasizing the importance of dynamic four-dimensional imaging of individual alveolar air spaces. While interpretation of these results must be taken with the experimental limitations in mind, the results do provide unique insight into alveolar dynamics within an \textit{in-vivo} large animal model.

Acknowledgments

The authors thank Luis Guerrero for his help with the surgical procedures, Elk Halpern for his help with the statistical analysis, and Alex Chee, Lida Hariri, and Melissa Suter for
discussions on lung physiology and pathology. This work has been supported by Air Liquide, Medical Gases, Les-Loges-en-Josas, France.