Synchrotron x-ray imaging of pulmonary alveoli in respiration in live intact mice

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Despite nearly a half century of studies, it has not been fully understood how pulmonary alveoli, the elementary gas exchange units in mammalian lungs, inflate and deflate during respiration. Understanding alveolar dynamics is crucial for treating patients with pulmonary diseases. In-vivo, real-time visualization of the alveoli during respiration has been hampered by active lung movement. Previous studies have been therefore limited to alveoli at lung apices or subpleural alveoli under open thorax conditions. Here we report direct and real-time visualization of alveoli of live intact mice during respiration using tracking X-ray microscopy. Our studies, for the first time, determine the alveolar size of normal mice in respiration without positive end expiratory pressure as 58 ± 14 (mean ± s.d.) μm on average, accurately measured in the lung bases as well as the apices. Individual alveoli of normal lungs clearly show heterogeneous inflation from zero to ~25% (6.7 ± 4.7% (mean ± s.d.)) in size. The degree of inflation is higher in the lung bases (8.7 ± 4.3% (mean ± s.d.)) than in the apices (5.7 ± 3.2% (mean ± s.d.)). The fraction of the total tidal volume allocated for alveolar inflation is 34 ± 3.8% (mean ± s.e.m). This study contributes to the better understanding of alveolar dynamics and helps to develop potential treatment options for pulmonary diseases.

Pulmonary alveoli, the elementary gas exchange units of the mammalian lungs, continuously inflate and deflate during respiration. This dynamic behavior of the alveoli significantly influences pulmonary function and stability1–3. Understanding alveolar dynamics is therefore crucial not only for studying emphysema or pulmonary edema, but also for treating patients with a variety of pulmonary diseases, such as acute respiratory distress syndrome (ARDS)4–5, that is a severe form of acute lung injury resulting from sepsis, trauma, or severe pulmonary infections. Patients suffering from these diseases are treated with mechanical ventilation, which eventually has negative side-effects on the lungs, including ventilator induced/associated lung injury (VILI/VALI)6–7. The visualization of alveolar dynamics has been, however, hampered by active lung movement during respiration.

The average size of the alveoli in live mammalian lungs (which is affected by the changing lung volume associated with breathing) remains undetermined, although this information is fundamental for understanding alveolar dynamics. When mice are sacrificed and the lungs are excised, the size of alveoli undergoes significant changes due to the drastically altered conditions (e.g., intra-thoracic pressure change, removal of surfactant, etc.)8–9. Moreover, it is not known how much individual alveoli inflate during respiration and whether the inflation is homogeneous or heterogeneous in live, breathing mammalian lungs. In addition, the fraction of the total tidal volume allocated for the inflation of the alveoli versus the non-alveolar parts of the lungs (e.g., alveolar central ducts) remains undetermined10–13, although these data would be important for determining the optimal tidal volume when treating ARDS patients with mechanical ventilation.

Real-time imaging of the alveoli is essential for determining the alveolar dynamics during respiration but it has been hindered by active lung movement14. Recently, subpleural alveolar clusters in live mice have been studied using intravital microscopy (IVM), optical coherence tomography (OCT), and optical frequency domain imaging (OFDI) under open thorax conditions15–17. However, in these studies the alveolar dynamics could be significantly affected by the intrathoracic pressure change once the thorax was opened16–18. Very recently, alveoli at the upper right lung apices that have a minimum lung movement were studied in live intact mice using tracking X-ray
microscopy (TrXM)\(^1\). However, real-time imaging of alveoli in any
other lung regions, in particular, at the lung bases in live intact mice
has not been done to date owing to the large respiratory motion.
In this study, we investigate alveolar dynamics not only in the lung
apices but also in the bases in live intact mice during respiration,
using tracking X-ray microscopy (TrXM II). X-ray imaging based on
phase contrast and strongly collimated synchrotron X-rays\(^2\)–\(^3\)
produces images of excellent quality due to strong edge enhancement
between different regions\(^2\)–\(^3\). Furthermore, synchrotron hard X-
rays are highly penetrating, enabling us to examine large (\(> 1 \times 1 \times 1 \) mm\(^3\)) regions\(^4\)–\(^5\) of the lungs, not limited to subpleural regions.
In addition, the projected radiographic images provide accurate
information on alveolar size, different from histological images of
sliced lung sections. The TrXM II method, which is capable of track-
ing individual alveoli despite large respiratory motion, allowed us to
directly measure the size and degree of inflation of individual alveoli
that were located in the lung bases as well as in the apices of live intact
mice during respiration.

**Results**

Visualization of alveoli described in a previous report\(^1\) was limited to
lung apices and it was not achieved in lung bases, basically due to the
active movement of the lungs, which was so big at this lung region
that the tracked alveoli were repeatedly in and out of the given field-
of-view (FOV) during respiration. For visualization of the alveoli in
the bases, therefore, the FOV should be enlarged beyond the move-
ment range of the bases. At the same time, high spatial and temporal
resolutions are required to resolve and track the small alveoli in rapid
movement during respiration.

In the present study, we developed TrXM II (Fig. 1a and b) that
allowed us to visualize individual alveoli not only in the lung apices
with minimum movement but also in the lung bases with maximum
movement (Fig. 1c) during respiration in live intact mice. The key
idea was to enlarge FOV to \(1 \times 1 \) mm, in order to amply cover
the area of maximum movements of the bases while providing the
high spatial and temporal resolutions of 500 nm (effective pixel size)
and 8 ms, respectively, to resolve and track individual alveoli during
respiration. Realization of TrXM II was based on a significant
improvement in the X-ray imaging detector system (Fig. 1b, blue
box in Fig. 1a; see Methods).

Figure 1d shows representative microradiographs of the right
upper (RU) lung apex (yellow) (top panels) and the left lower lung base (yellow) (bottom panels) in a live intact mouse, taken at the ends of expiration and inspiration with a
normal tidal volume (160 \(\mu\)l) (Supplementary Video 1).

**Figure 1** | Tracking X-ray microscopy II. (a) Schematic of tracking X-ray microscopy II. The sample stage, a fast shutter, and an X-ray imaging detector
system (blue box) were synchronized to a mechanical ventilator. SR: Synchrotron Radiation, M(left): Monochromator, FS: Fast Shutter, V: Ventilator, S:
Scintillator, M: Mirror. (b) Schematic of the X-ray imaging detector system. Using a high light yield scintillator of LSO:Tb/LYSO:Ce (4 mm \(\times\) 4 mm,
3.2 \(\mu\)m thickness), X-ray images were efficiently converted to visible ones. The visible images were then reflected by a small right angle mirror (3 mm
height) and magnified by a 20× objective lens coupled with a tube lens for aberration correction before captured by a CMOS camera. (c) Schematic of
overall lung movements in the apex top and the base bottom during respiration. (d) Representative microradiographs of the right upper lung apex
(yellow) (top panels) and the left lower lung base (yellow) (bottom panels) in a live intact mouse, taken at the ends of expiration and inspiration with a
normal tidal volume (160 \(\mu\)l) (Supplementary Video 1).
by the interference effect from their different movement directions during respiration. This finding indicated that the spatial and temporal resolutions (500 nm and 8 ms, respectively) were high enough to directly measure in real-time the size and the degree of inflation of individual alveoli in the lung bases during respiration.

Figure 3 shows alveolar size (at the end of expiration) and alveolar inflation, measured from real-time 2-D microradiographs [see Methods], for the RU (a) and the left upper (LU) (b) apices, and the right lower (RL) (c) and the LL (d) bases. To confirm the validity of our 2-D measurements of alveoli in respiration, we carried out real-time microtomography of the alveoli in respiration in a live intact mouse. The microtomography enabled us to measure the size of individual alveoli of the mouse in 3-D geometry, as demonstrated on Supplementary Fig. 1. Comparison of the alveolar sizes measured in 2-D and 3-D showed a good correlation within 99 ± 2% (Supplementary Fig. 1d), indicating reasonable validity of the 2-D measurements. For statistical analysis, the 2-D measurements were done for 24 alveoli per mouse (4 sites) for 5 mice (Table 1). As seen on the plot of the data for all the regions on Fig. 3e, we found that the average alveolar size of live intact mice was 58 ± 14 (mean ± s.d.) μm (the vertical navy line). The size was on average a little larger in the RL bases (64 ± 14 (mean ± s.d.) μm) than in the RU apices (54 ± 10 (mean ± s.d.) μm) (Table 1 and Fig. 3a–d, P = 0.002).

Interestingly, the individual alveoli in normal mice show heterogeneous inflation from zero to ~25% in size (Fig. 3a–e; Supplementary Fig. 2). The average inflation, which is 6.7 ± 4.7% (mean ± s.d.) for all (RU, LU, RL, and LL) regions (the horizontal brown line on Fig. 3e), slightly increases from 5.7 ± 3.4 (3.2)% (mean ± s.d.) on the RU (LU) apices to 6.9 ± 4.3% (mean ± s.d., P = 0.261) for the RL bases, and to 8.7 ± 6.6% (mean ± s.d., P = 0.033) for the LL bases.

Depending on the lung region, the lung movement is significantly different. The smallest is for RU (86 ± 18 μm (mean ± s.e.m.)), increased for LU (214 ± 43 μm (mean ± s.e.m.)) and RL (423 ± 67 μm (mean ± s.e.m.)), and the largest is for LL (513 ± 76 μm (mean ± s.e.m.)), as demonstrated by the regional displacements of the pleural surfaces during respiration in Fig. 4a. The alveolar expansion (Fig. 4b), expressed by absolute expansion of individual alveoli, seems to be influenced by the regional differences in lung movement, increasing from 3.0 ± 0.3 μm (mean ± s.e.m.) in the upper apices to 4.3 ± 0.5 μm (mean ± s.e.m., P = 0.034) in the RL bases and further to 5.5 ± 0.9 μm (mean ± s.e.m., P = 0.014) in the LL bases.

**Discussion**

Despite recent improvements in the experimental techniques used for studying alveolar dynamics, previous works have been limited to studying alveoli at the right upper lung apices that have minimal lung movement, subpleural alveoli under open thorax conditions, or alveoli that have been removed from the body and processed for histology. The strength of TrXM II imaging we use in this study is that it is applicable to dynamic studies of thick living organs in active movements. At the same time it has high spatial and temporal resolution. Here we demonstrate that the high penetrating power of synchrotron hard X-rays enabled us to directly visualize and accurately measure individual alveoli in live intact mice not only in the lung apices but also at the basal lung area during ventilation.

Many reports have been published stating that the lung behaves in a geometrically similar fashion (e.g., Ardila et al., 1974; Weibel 198627–28), but all these studies were done in a macroscopic scale. In other words, the data of these publications do not necessarily indicate that the alveolar structure behaves in an isotropic fashion (e.g., Greaves et al., 198612). In fact, we found a large variation, 70% (s.d./mean of Fig. 3e), in alveolar inflation. In other words, even exposed to the same pressure, the inflation of the neighboring alveoli is quite different; this indicates substantial asynchrony at the acinar level. Second, in addition to the significant variations in the degree of alveolar inflation (Fig. 3e), the alveolar duct (which is usually considered to consist of two parts; the central channel and sidewall alveolar pockets) does not appear to expand in an isotropic fashion. Assuming an alveolus can be treated as a 65% (mean ± s.e.m.) truncated sphere with a sphericity of 0.931 ± 0.022 (mean ± s.e.m. (Fig. 3b of Chang et al., 201319), alveolar volumes at the ends of expiration and inspiration were estimated (Supplementary Table 1a) based on the measured diameters (Table 1). Furthermore, the average volume changes of alveoli of four lung regions were calculated from the volume difference between inspiration and expiration (Supplementary Table 1b); averaging the values over four lung regions, overall average of alveolar volume change was calculated as ∆Vave = 1.8 ± 0.2 × 10−7 μL. (see Supplementary Table 1 for the average volume change of 120 individual alveoli measured over four lung regions in 5 mice). If we assume that the total number of alveoli in mice weighing 22.5 g is about 3 million29–32, the volume change of the sidewall alveolar pockets would be 54 ± 6 μL (mean ± s.e.m.), which is 34 ± 3.8% (mean ± s.e.m.) of the tidal volume (160 μL). The rest (66%) of the tidal volume is accounted for by central channel expansion. The fact that there is a substantial difference in

![Figure 2](https://www.nature.com/scientificreports/) Tracking individual alveoli in the lung base. (a) Schematic of overall lung movement. (b) Representative microradiographs of the lower left lung base (black box of (a)) of a mouse during one normal inspiration (200 ms)—expiration (400 ms) cycle (0, 200, 400, and 600 ms from the left). (c) The magnified regions correspond to blue boxes in (b). Three trackable alveoli (red, orange and blue dashed circles) are demonstrated.
alveolar expansion and central channel expansion indicates that the lung does not expand isotropically at the level of alveolar duct; these data clearly support the observation that the lungs often change their volume but with small change in surface area (e.g., Bachofen et al., 1987; Sera et al., 2013; Fig. 1C of Greaves et al., 1986).

A substantial asynchrony at the level of the alveoli must have a significant effect on gas exchange and aerosol mixing and deposition. For instance, since the alveolar airflow is chaotic, we demonstrated that 10% asynchrony (Miki et al., 1993) can induce a substantial airflow mixing. Thus, we expect that an even larger variation in alveolar inflation (70%, Fig. 3e) as well as a substantial difference in alveolus/duct expansion could cause significant airflow mixing at the alveolar level enhancing gas exchange and aerosol deposition.

The degree of lung displacement during respiration is presumably associated with the anatomical space available for lung expansion in each anatomical region of the chest of the mouse. As the apical regions are tightly confined by the rib cage, the displacements of the apical regions are small (Fig. 4a). On the other hand, the large movements of the lung at the base are mostly attributed to the active movement of the diaphragm. A slightly larger displacement in the LL than in RL is consistent with this idea, since the motion of the left hemidiaphragm is generally greater than that of the right hemidiaphragm.

In this study, we successfully applied a novel technology for in vivo, non-invasive visualization and quantification of alveolar size in live intact mice using TrXM II that enabled tracking individual alveoli in active movements. TrXM-based identification of alveolar dynamics in the RU and LU lung apices and the RL and LL bases revealed that the average alveolar size was 58 ± 14 μm (mean ± s.d.) at functional residual capacity (FRC) and heterogeneous alveolar inflation by 6.7 ± 4.7% (mean ± s.d.) during respiration. The fraction of the total tidal volume allocated for alveolar inflation was estimated as 34 ± 3.8% (mean ± s.e.m.). TrXM analysis of the alveoli can open the way to various studies of alveolar dynamics in live intact animals, with future promise to contribute to a better understanding of emphysema and pulmonary edema, and other diseases related to alveolar dysfunction, for instance, VILI in ARDS.

Table 1 | Alveolar size (the major axis of each alveolus) at the ends of expiration and inspiration and alveolar inflation of each region with standard deviation (s.d.). 24 alveoli per mouse (4 sites, n = 5), i.e. a total of 120 alveoli were measured.

| Region of Lung | Expiration (mean ± s.d. μm) | Inspiration (mean ± s.d. μm) | Alveolar inflation (mean ± s.d. %) |
|----------------|-----------------------------|-------------------------------|----------------------------------|
| Apex of Upper Lung | 54 ± 10                    | 57 ± 10                       | Apex of Upper Lung 5.7 ± 3.4     |
| Base of Lower Lung | 64 ± 14                    | 60 ± 15                       | Base of Lower Lung 6.9 ± 4.3     |

Methods

The methods were carried out in accordance with the approved guidelines.

Animal preparation. All experimental protocols were approved by the SPring-8 Experimental Animals Care and Use Committee. Eight-week-old SPF pathogen-free nude mice (BALB/c-nu, body weight: 20–25 g, male, SLC Japan Inc., Japan) were examined. For anesthesia, sodium pentobarbital (50 mg kg−1) was injected into peritoneum. Tracheotomy was then performed, followed by the insertion of a catheter (22 G JELCO® I.V. Johnson & Johnson Medical, TX, USA). Ventilation of mice was carried out with room air via the catheter using a ventilator (Inspira-Advanced Safety Ventilator-Pressure Controlled (ASVP), Harvard Apparatus, USA). Physiological respiratory conditions were chosen with an inspiration/expiration ratio of 1:2, a tidal volume of 160 μL/respiration, and a respiratory rate of 100 breaths/min without positive end expiratory pressure. Each mouse was placed in an acrylic tube and fastened in a vertical position for X-ray imaging. After the imaging experiments, all the mice were alive.
Measurement of alveolar size. Alveolar size was measured both at the end of expiration and at the end of inspiration from 2-D microradiographs. Individual alveoli were first identified with their individual movement paths in 2-D microradiographs during expiration similar to our previous study46. Alveolar size was defined here as the maximum diameter of an alveolus, as demonstrated by an orange arrow in Supplementary Fig. 1c. Alveolar inflation is defined as the percentage increase by inspiration.

To confirm the validity of 2-D measurement, the alveolar sizes measured in 2-D and 3-D images were compared. For this, microtomography was performed for a right lower base, using TrXM II with a high-sensitivity camera (PCO.edge, PCO Imaging, Germany), with an exposure time of 20 ms and a resolution of 590 nm per pixel. Each image was taken at the end of expiration during 180° rotation (Supplementary Movie 2). 20 alveolar sizes were measured from 3-D volume-rendered images (Supplementary Fig. 1b) and from 2-D microradiographs (Supplementary Fig. 1c) for identical alveoli, as demonstrated in Supplementary Fig. 1b (an orange arrow) and c (an orange arrow), respectively, for the corresponding alveolus, marked by the arrow head in Supplementary Fig. 1a. A good correlation within 99 ± 2%, calculated by averaging the ratio between the 2D-based size and the 3D-based size, is demonstrated in Supplementary Fig. 1d.

**Statistical analysis.** Data were presented as mean ± s.d. or mean ± s.e.m. P-values were determined by performing a two-tailed t-test with data obtained from the right upper apices.

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**Author contributions**

S.C., N.K. and J.K. performed experimental work and data analyses. Y.K. and T.I. supported to run synchrotron X-ray imaging experiments in SPring-8. C.K.R. and A.T. performed data analysis and interpretation. J.H.J. supervised the work. J.H.J. and C.K.R. helped to design the experiments. S.C., N.K., J.H.J. and A.T. wrote the manuscript and prepared figures 1–4. All authors reviewed and edited the manuscript.

**Additional information**

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