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Visualizing treatment delivery and deposition in mouse lungs using *in vivo* x-ray imaging

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Abstract

The complexity of lung diseases makes pre-clinical *in vivo* respiratory research in mouse lungs of great importance for a better understanding of physiology and therapeutic effects. Synchrotron-based imaging has been successfully applied to lung research studies, however longitudinal studies can be difficult to perform due to limited facility access. Laboratory-based x-ray sources, such as inverse Compton x-ray sources, remove this access limitation and open up new possibilities for pre-clinical small-animal lung research at high spatial and temporal resolution. The *in vivo* visualization of drug deposition in mouse lungs is of interest, particularly in longitudinal research, because the therapeutic outcome is not only dependent on the delivered dose of the drug, but also on the spatial distribution of the drug. An additional advantage of this approach, when compared to other imaging techniques, is that anatomic and dynamic information is collected simultaneously. Here we report the use of dynamic x-ray phase-contrast imaging to observe pulmonary drug delivery via liquid instillation, and by inhalation of micro-droplets. Different liquid volumes ($4\,\mu l$, $20\,\mu l$, $50\,\mu l$) were tested and a range of localized and global distributions were observed with a temporal resolution of up to 1.5 fps. The *in vivo* imaging results were confirmed by *ex vivo* x-ray and fluorescence imaging. This ability to visualize pulmonary substance deposition in live small animals has provided a better understanding of the two key methods of delivery; instillation and nebulization.

Keywords: x-ray imaging, *in vivo* small animal imaging, treatment delivery, lung imaging, fluorescence imaging

1. Introduction

There is no established *in vivo* method to directly observe the delivery of a treatment into the lungs, or the resulting treatment distribution. In this report, lung imaging possibilities are discussed and an *in vivo* method for observing the process of liquid instillation or aerosol inhalation for pulmonary application of drugs in small animals is presented, using dynamic x-ray phase-contrast imaging.

The instillation or inhalation of liquid to the lungs of small animal models can be used to generate infections (e.g. *pseudomonas aeruginosa* for cystic fibrosis) or to mimic specific illnesses

(e.g. *bleomycine* for pulmonary fibrosis), and to test respiratory treatments. Clinically, the most widely-used inhaled drugs are corticosteroids and bronchodilating drugs, but more recently patients with pulmonary infections are often treated using inhaled antibiotics. The success of a treatment is dependent on achieving the correct local concentrations of the deposited agents, and unfortunately more treatment often deposits in the healthy regions of the lung than the infected part of the lung [1, 2, 3, 4]. Liquid dosing is commonly used in animal-based gene transfer studies, however gene expression is often variable, potentially due to the different liquid distributions in the lungs of each animal [5]. For aerosolized drug application, the expected pulmonary distribution of the drug is often derived from computational aerosol-lung deposition models, which take into account particle-related factors, such as particle size and shape, as well as patient-related factors, such as airway size, health and the quality of inhalation [2, 6]. However, these models need to be validated using experimental measurements, and while there are a suite of methods used to take measurements of flow and deposition in models of airways and in animals [7], existing methods are typically impossible to perform *in vivo*, or lack spatial resolution. The x-ray methods we propose here may therefore assist in verifying the accuracy of computational aerosol-lung deposition models, and be used in pre-clinical treatment trials with small animal models to correlate the treatment effectiveness with the local deposition of the treatment.

The lungs are a challenging organ to image, and a number of approaches have been used over the years. Perhaps the most significant challenge is the constant motion of the lungs throughout the breathing cycle, which can lead to motion artifacts that reduce the spatial resolution of the image. In addition, the lung often suffers from low image contrast. Here, we briefly discuss the structural and functional imaging methods most commonly used to reveal lung anatomy and physiology. The fastest, simplest, and lowest-cost method by which to image lung structure is a standard diagnostic x-ray radiograph. Although chest x-rays are a routine examination, the images suffer from low contrast as the x-ray absorption coefficient is very similar for both air and soft tissue. In the clinic, this limits the use of chest x-rays to the diagnosis and monitoring of respiratory conditions such as pneumonia, emphysema, pneumothorax, and some lung cancers. Another lung imaging option is computed tomography (CT), which can capture virtual slices through the lung and therefore better differentiates the lung from surrounding anatomy than a projection radiograph. However, CT is most straight-forward with stationary samples, without any motion reducing the spatial resolution as a result of motion artifacts [8]. Magnetic resonance imaging

(MRI) is a challenging method for morphological imaging of the lung, since the MR image signal arises from hydrogen atoms, and the lung consists mostly of air. Furthermore, the multiple air-tissue interfaces create image artifacts. MRI acquisition is relatively slow, so lung images can suffer from respiratory motion artifacts that lead to poor image quality [9, 10, 11].

In contrast to these structural imaging techniques, functional imaging is commonly performed using nuclear methods such as positron emission tomography (PET) or gamma scintigraphy. These techniques are used in studies of lung physiology and metabolism, including regional perfusion, gene expression, enzyme and receptor studies [12]. To obtain a signal, a radio-labeled substance must be administered, and its radioactive decay can then be detected. While those techniques can localize radiotracer fluids, none have the spatial or temporal resolution to do this within a breath and neither provide any anatomical information, which often makes it necessary to acquire a corresponding image using CT or MRI [13, 1, 14].

The challenge we address in this report is the acquisition of images with a sufficient spatial and temporal resolution to observe inhaled and instilled liquid delivery, distribution, and deposition in the lungs of small animal models. As discussed above, with most conventional imaging methods the lung produces only low contrast, but phase-contrast x-ray imaging (PCXI) techniques can be used to enhance the lung contrast. We achieve this in a laboratory environment, using propagation-based PCXI at a compact synchrotron light source. Phase contrast originates from the refraction of the x-rays, i.e. the gradient of the x-ray wavefield's phase, rather than absorption. The advantage of phase-contrast imaging is that the phase gradient, and hence the contrast, is largest at the interfaces between different materials, which leads to an edge-enhancing effect. The lung is an ideal sample for phase-contrast x-ray imaging as it consists of many air-tissue interfaces and the difference in the x-ray refractive index between the air and tissues is large, resulting in steep x-ray phase gradients and strong contrast at air/tissue interfaces. A number of x-ray phase imaging techniques have arisen in the last couple of years, all of which can dramatically enhance soft tissue contrast. The most widely-adopted approaches are grating interferometry [15, 16] and propagation-based phase-contrast imaging [17, 18]. Both methods were developed at synchrotron facilities, where high spatial and temporal coherence results in easily-observable phase effects. While grating-based PCXI is well-established in laboratory environments, propagation-based imaging is still most often used at synchrotron sources, because high spatial coherence is required to observe these phase effects. Grating interferometry can avoid

the requirement for high spatial coherence by inserting a grating, typically in front of the source [19]. While grating interferometry delivers sensitive differential phase, absorption and dark-field images, the temporal resolution is low, as a phase-stepping scan (ie. multiple exposures) is necessary to reconstruct an image. The only exception to this limitation is when a motion is repeated, such as the breath cycle, presenting multiple opportunities to capture the sample in the same position [20]. A dark-field signal is generated when sub-pixel structures scatter the x-ray wavefield, and the strength of this signal will depend on the number and size of scattering structures [21, 22]. As a result of the many scattering structures in the lung the technique can provide high contrast lung images, provided the temporal resolution is not important [23, 24, 25, 26, 27], making some clinical applications feasible [28, 29]. We considered if there was potential for the dark-field modality to reveal the presence of treatment, noting that the treatment delivery is not a repeated motion. In order for the presence of treatment in the lungs to change the dark-field signal, the treatment would need to sufficiently fill the air sacs in the lung to change the effective size of those air sacs. We experimentally tested this approach by imaging an instillation process into the lung with grating-based imaging, however the temporal and spatial resolution was too poor to produce satisfying results, so it is not reported here.

For dynamic imaging of a once-off event like treatment delivery, propagation-based imaging is more suitable as the image is generated in a single exposure and no additional optical elements are introduced, maximizing the use of the available x-ray flux. The resulting image includes both attenuation contrast, which reveals bones and strongly-absorbing liquid like iodine, and edge-enhancing phase effects, which introduce bright/dark lines at each air/tissue interface. However, the beam coherence requirements for propagation-based imaging are more strict than for grating-based imaging, so in a laboratory environment the technique typically uses a micro-focus source [30, 31], and the associated long exposure times limit applications to static samples. For this reason most studies that perform dynamic x-ray lung imaging have been performed at high-flux synchrotron facilities [32, 33, 34, 35, 36, 37, 1, 38]. However, these facilities are expensive, rarely designed for small-animal imaging, and access is very limited, which can prohibit longitudinal experiments, or time-critical imaging (i.e. cancer models).

To make phase-contrast imaging widely accessible, it is necessary to bring the technique into the laboratory. The recent development of novel high-flux and high-coherence laboratory x-ray sources, including liquid-metal-jet sources [39] and inverse Compton sources [40, 41, 42,

43, 44], mean that the widespread use of dynamic propagation-based imaging is moving closer to a reality. For example, Larsson et al. used a liquid-metal-jet x-ray source to capture phase-contrast tomographic images of an excised air-filled mouse lung [45], Preissner et al. captured *in vivo* mouse lung structure [46], and Kim et al. performed 4DCT measurements at a liquid-metal-jet source to explore the effect of mechanical ventilation on the lung [47]. Using an inverse Compton source, we have recently shown *in vivo* PCXI of mouse respiratory function [48]. The advantages of a Compact Light Source are quasi-monochromatic x-rays, and relatively high coherence and high flux, all of which makes such a source suitable for dynamic propagation-based x-ray phase contrast imaging [41].

This report describes the non-invasive dynamic *in vivo* imaging of liquid deposition during and immediately after delivery into the lungs of mice, using laboratory-based PCXI. The liquid was either instilled directly into the trachea, or inhaled via an aerosoliser attached to a small animal ventilator system. The temporal and spatial resolution was sufficient to visualize the dynamics of the fluid delivery entering the lungs and then distributing throughout the airways, with results covering a range of treatment distributions. This is the first time that such dynamics have been visualized by x-ray imaging in a laboratory environment.

2. Materials and Methods

2.1. In vivo imaging setup

Imaging was performed at the Munich Compact Light Source (MuCLS) at the Technical University of Munich (TUM) in Garching, Germany. The MuCLS consists of an inverse Compton x-ray source (Lyncean Technologies Inc., Fremont, USA), which delivers tunable quasi-monochromatic x-rays between 15 keV and 35 keV and x-ray beamline infrastructure designed and installed by TUM researchers. A detailed description of the working principles has been published [43, 49, 50, 51]. For all experiments a quasi-monochromatic 25 keV x-ray beam was used, with flux up to $2 \cdot 10^{10}$ ph/s and a divergence of 4 mrad. Mice were positioned vertically on a remotely controlled x-y-z-rotation stage 4 m downstream from the x-ray source. At the sample position, the x-ray beam has a diameter of about 16 mm. The sample-to-detector distance was 1 m (geometric magnification M = 1.25). Images were captured with a $20\,\mu m$ thick Gadox scintillator (Gd_2O_2S:Tb) (CRYTUR, spol. s r.o., Czech Republic) deposited on a 2:1 fiber optic

taper coupled to an Andor Zyla 5.5 sCMOS camera, resulting in a detector pixel size of $13\,\mu m$ (effective pixel size $p_{eff} = 10.4\,\mu m$). The CCD-detector had an array size of 2560×2160 pixels. The surface entrance dose was estimated to 2 mGy for a 100 ms x-ray exposure at 25 keV. This estimation relies on measurements of the air kerma with an ion chamber which was placed 7.2 m from the interaction point (a 1.5 cm thick PMMA block was used as a backscattering object), with these values converted to absorbed dose for water (surface entrance dose), which is comparable to radiation doses used in *in vivo* small animal synchrotron and micro-CT imaging [52, 53, 54, 55].

Figure 1: (a) Setup for imaging of liquids applied to the lungs of ventilated mice. The syringe pump was remotely actuated to deliver a preset volume of liquid through a thin tube inserted into the lung through the intubation cannula of the ventilator circuit. (b) Raw image of a $50\,\mu l$ instillation of Liquid I, captured 13 s after delivery began. (c) Difference image produced by subtracting the pre-delivery image, which makes the liquid distribution clearly visible. (d) A pseudo-colored image, which is a composition of b and c (with the same color maps), showing the surrounding anatomy and the treatment deposition. Scale bar: (b) 2 mm

2.2. Animal Handling

All procedures for animal handling and experiments were performed in accordance with protocols approved by the Regierung von Oberbayern (District Government of Upper Bavaria, AZ55.2-1-54-2532-108.13), with female C57BL/6 mice (8 - 18 weeks old; 20 - 24 g body weight). Mice were anaesthetized by intraperitoneal injection of a mixture of Medetomidine (0.5 mg/kg body mass), Midazolam (5.0 mg/kg body mass) and Fentanyl (0.05 mg/kg body weight). Animals were then intratracheally intubated using a non-surgical technique [56, 5] with a 20 Ga intravenous catheter used as an endotracheal cannula. After intubation, the animal was placed into a custom designed imaging holder and positioned for imaging in the x-ray radiation enclosure.

2.3. Treatment preparation

The treatments consisted of a mixture of a clinical iodine-based contrast agent (Ultravist-370, 370 mg iodine/ml) and a red-active fluorescent dye (Sky Blue (ex/em = 670 nm/710 nm, Kisker Biotech GmbH, Steinfurt, Germany)), which contains fluorescent polystyrene particles (1% w/v,

diameter 480 nm) dispersed in distilled water. Iodine acted as a contrast agent for x-ray imaging and Sky Blue was used for fluorescence analysis in the *ex vivo* lungs, with the mixture providing the ability to compare the fluid deposition with two different imaging modalities. Two different mixtures of these agents were used: Liquid I was a mixture of 0.5 ml Sky Blue, 2.5 ml Ultravist-370, and 7 ml water, and was used for the instillation experiments. Liquid II consisted of 0.3 ml Sky Blue, 0.9 ml Ultravist-370 and 3.6 ml water, and was used for the aerosol inhalation experiments.

2.4. Instillation

The setup for instillation of a liquid into the mouse lungs is shown in Fig. 1 (a), which was similar to that described in Donnelley et al. [36, 37]. The intra-tracheally intubated mice were mechanically ventilated throughout the delivery and imaging period using a flexiVent small-animal ventilator (flexiVent FX, Scireq, Montreal, QC, Canada). The ventilation protocol was set to a respiratory rate of 90 breaths/min, with a breath-hold (222 ms) at the end of the inhalation phase (222 ms) and prior to the expiratory period (222 ms), with a tidal volume of 30 ml/kg and 30 cm H_2O maximum pressure limit. Image acquisition was triggered at the end of the inhalation phase, so that the exposure took place during the breath-hold, resulting in a frame rate of 1.5 fps. Liquid I was introduced via a heat thinned PE10 polyethylene tube (< 0.5 mm diameter) that was passed through the flexible wall of the ventilator inspiratory tube (see Fig. 1 (a) (red box)). The PE tube was small enough to not block the intubation cannula, but made it possible to simultaneously deliver the liquid and maintain ventilation. A remotely controlled syringe pump (UltraMicroPump III and Micro4 controller, World Precision Instruments, Sarasota, FL) allowed for continuous imaging throughout the liquid delivery.

Either $4\,\mu l$ or $50\,\mu l$ of Liquid I was instilled by operating the syringe pump at a continuous flow rate of $0.8\,\mu l/s$ or $4.0\,\mu l/s$, resulting in delivery times of 5 s and 12.5 s, respectively. These were slower than a typical fast application (< 1 s) where a loaded syringe is emptied via the intubation cannula into the trachea. Image acquisition (200 ms) was triggered by the ventilator during the end-inspiratory breath hold (at total lung capacity) (Fig. 1, Fig. 2, Fig. 3 (a,b)), which reduced motion artifacts and allowed frame rates of up to 1.5 fps.

In a separate set of instillation experiments with spontaneously breathing mice (Fig. 3 (c)), the ventilator was replaced by a non-contact fibre optic displacement sensor (RC-60, Philtec, MD) that was configured to detect respiratory motion and deliver an electrical trigger signal for image capture at the same point of each breath [58]. The frame rate was maintained at approximately 1.5 fps, but the exposure time was reduced to 100 ms to mitigate motion blur as induced by non-steady lung inflation during spontaneous breathing. These animals received a volume of $4\,\mu l$ within 5 s (delivery rate $0.8\,\mu l/s$) using the instillation setup described above.

2.5. Inhalation

Liquid II was used for the aerosol inhalation experiments (see section 2.3). The flexiVent small animal ventilator was operated in the same way as during the instillation experiments, with a respiratory rate of 80 breaths/min including a breath hold (204.3 ms) at the end of the inspiratory period (297.5 ms) and prior to the expiratory period (225.0 ms), resulting in 1.33 fps. An Aeroneb aerosol generator (Aerogen Inc., Galway, Ireland) was placed into the ventilator inspiratory line. The Aeroneb uses a vibrating mesh to aerosolize small quantities of liquid, with a liquid output rate of 0.36 ml/min and a droplet diameter of $3.6\,\mu m$ (white light scattering; INAS 100, Palas, Karlsruhe, Germany). During inhalation, up to $150\,\mu l$ of liquid could be nebulized without cleaning the tubing, but longer inhalation periods (and therefore larger aerosol volumes) would block the tubing with droplets. For a nebulizer on-time of 40 ms per breath, $150\,\mu l$ was nebulized within ~ 11 min, but imaging was continued for a total period of 14 min, corresponding to 1120 breaths. After each run the tubing of the ventilator was dried, and the Aeroneb was cleaned and refilled with Liquid II. In some cases a second nebulization with $75\,\mu l$ or $150\,\mu l$ was performed yielding 225 and $300\,\mu l$ of total nebulized liquid, respectively. Due to aerosol deposition within the ventilator circuit, and partial aerosol deposition in the lung, only 12 to $24\,\mu l$ of the 150 to $300\,\mu l$ aerosolized liquid was deposited in the lung as determined by *ex vivo* epifluorescence imaging (as described below).

2.6. Image analyses

To generate the difference images (Fig. 1 (c)), we considered the line integrals of the attenuated

intensity (Fig. 1 (b)), following Lambert's law,

$$-\ln((I_{Mouse}+I_{Liquid})/I_0)=\mu_M\times T_M+\mu_L\times T_L, \quad (1)$$

where I_0 is the intensity of the incident x-rays, I_{Mouse} is the intensity of the transmitted x-rays before delivery and $I_{Mouse}+I_{Liquid}$ is the intensity of the transmitted x-rays during and after delivery. μ_M and μ_L are the linear attenuation coefficients for the mouse and the liquid respectively, with the corresponding projected thicknesses T_M and T_L.

A pre-delivery image was subtracted from the sequence to isolate the treatment/liquid effects,

$$-\ln(I_{Mouse}+I_{Liquid})/I_0)+\ln(I_{Mouse}/I_0)=\mu_L\times T_L. \quad (2)$$

To generate the pseudo-color images (Fig. 1 (d)), a threshold and a binary opening filter was used on the difference images to reduce artificial signals (e.g. due to respiratory movements the edges of the lung and the speckles that arise from scattering from the aveoli are never in exactly at the same place in each image, and hence could produce some signal in the difference image). In order to display the difference images, a heat-map was chosen. The pseudo-color image was generated by combining the colored difference image (from Eqn. 2, Fig. 1c) with the corresponding integrated image (Eqn. 1, Fig. 1b). The Python programming language (Python Software Foundation) [59, 60] was used for the image analysis. Each lung shown in Fig. 3 was analyzed regionally by selecting one or more regions of interest (ROIs) in the difference images. The ROIs are displayed in the pseudo-colored images in Fig. 4 (a)-(c). The fraction of total delivery plotted over time in Fig. 4 (d)-(f) is the sum of $\mu_L T_L$ in a region of interest (e.g. i pixels) at a given time point t divided by the sum of $\mu_L T_L$ values of the whole lung area (e.g. j pixels) in the last frame of the sequence:

$$\text{fraction of total delivery}_t=\frac{\left[\sum_i(\mu_L T_L)_i\right]_t}{\sum_j(\mu_L T_L)_j}\times 100 \quad [\%]. \quad (3)$$

Figure 2: Comparison of *in vivo* and *ex vivo* imaging modalities after intratracheal application of $50\,\mu l$ Liquid I, which preferentially targeted the right lung. (a) *In vivo* x-ray image with the liquid distribution presented in pseudo-color. (b) Coronal and sagital slice of the reconstructed volume from an *ex vivo* CT scan. The yellow line indicates the position of the sagital and coronal slices,

respectively. (c) Rendering of a tomographic reconstruction of the excised lung. A movie of the rendering is available in the supplementary material (Video 4). (d) Epifluorescence image of the whole *ex vivo* lung. (e) Photograph of an inflated dried *ex vivo* lung. (a)-(d) is one murine lung, (e) is a different lung. Scale bar: (a,b) 2 mm, (c) 2.5 mm

2.7. Ex vivo lung tomography

After the *in vivo* experiments, the lungs were immediately excised and dried while partially inflated at a pressure of 2 kPa (close to the end-inspiratory pressure) (see Fig. 2 (e)), according to the protocol described by Harbison et al. [16]. A tomography scan (CT) of the dried lung was then performed, capturing 2049 projections with an exposure time of 270 ms per projection, over 360 degrees. The images were reconstructed using a standard filtered back projection reconstruction algorithm (see Fig. 2 (b)). The three dimensional data-sets were rendered using AVIZO Fire 8.1 (Thermo Fischer Scientific Inc, Massachusetts, USA) (see Fig. 2 (c)).

2.8. Ex vivo fluorescence imaging

After CT imaging of the dried lungs, *ex vivo* epifluorescence images were obtained from the whole and sliced lung using an IVIS (In Vivo Imaging System, Lumina II, Caliper/Perkin Elmer, USA) equipped with an excitation / emission filter combination (ex/em = 640 nm/Cy5.5) suitable for the Sky Blue fluorescent dye (see Fig. 2 (d)). To determine the aerosol volume/dose deposited in the lungs, the dried lungs were sliced, imaged and analyzed for dosimetry similar to the method described by Barapatre et al [62]. For this, the dried lungs (partially inflated by application of a 2 kPa air pressure to the trachea during drying) were embedded in polyurethane foam (PU foam Pattex Ultraweiss, Pattex, Germany) for stabilization and subsequently cut into 1 mm thick slices perpendicular to the orientation of the trachea. An IVIS epifluorescence image was obtained from each slice and the total fluorescence intensity ("total radiance efficiency" (photons/s) / (μW-laser/cm^2)) of the lung was determined from the slice intensities. Using a standard curve obtained from lungs containing a known amount of dye (instillation of known amounts of dye into the lungs) this intensity was converted into an equivalent lung deposited aerosol volume (or dye mass / dose).

3. Results

3.1. Instillation

Fig. 1 (b) shows a typical x-ray attenuation image, corrected for illumination inhomogeneities and detector effects, and demonstrates that the periphery of the lung is clearly visible. It is evident that visualization of the delivered liquid was difficult, particularly in the case of overlying bones. The subtraction of the pre-delivery image (resulting in Fig. 1 (c)) much more clearly shows the liquid distribution. This method required that the chest anatomy remained unchanged during the entire longitudinal imaging process, which was accomplished by placing the sedated mouse into a specially designed holder and by taking the images during a breath hold at the same state of lung inflation (here: full inflation at the end of the inspiratory phase). By overlaying the 'normal' image (Fig. 1 (b), greyscale) with the difference image (Fig. 1 (c), colored), we generated a pseudo-color image (Fig. 1 (d)), showing where the treatment was present (primarily in the centrally located post-caval lobe). The entire time sequence of images for this delivery can be found in the supplementary material (Video 1). The validity of the assumption that the subtracted pre-delivery image remains mainly constant during the entire imaging period was confirmed by obtaining no significant difference signal for blank lungs (no liquid instillation or inhalation of aerosol; see supplementary material Video 2).

Figure 3: Pseudo-colored image sequences of three different mice show (a) an intentionally local and (b) a global distribution of 50 µl of Liquid I (see methods for detail) instilled within 12.5 s through the trachea and depositing in the lung. Row (c) shows a delivery of only 4 µl of Liquid I within 5 s. The sequences shown in panels (a) and (b) were captured when the mouse was ventilated, and panel (c) was captured while the mouse breathed freely (with the image triggered by chest motion). The numbers in the bottom part of each frame indicates the time in seconds after the liquid first appeared in the field of view (time was set to 0 s). The frames shown here were chosen to best illustrate the progression of the delivery. (d)-(f) show the corresponding fluorescence images of the whole *ex vivo* lung. Apart from panel (d), there are differences between the observed distribution in the fluorescence images and the *in vivo* x-ray image. This was likely due to light attenuation mitigating the fluorescence signal from deeper tissue, with *in vivo* images providing a 2D projection of the 3D drug distribution. Scale bars: all 2 mm.

Immediately after the *in vivo* experiments, the lungs were excised and dried while inflated [61]. Some of the dried inflated lungs were then scanned using computed tomography to capture the distribution of the instilled treatments in three dimensions. The reconstructed 3D tomography can then be compared to the *in vivo* 2D projections, as shown in Fig. 2. Fig. 2 (a) shows the pseudo-colored *in vivo* instillation image 18 s after the start of treatment delivery, and (b) a coronal and transverse slice of the CT volume of the dried lung.

Fig. 2 (c) shows the CT rendering of the *ex vivo* lung. The part of the rendered lung that is colored yellow had the highest absorption values, indicating that was the location where the bulk of the highly x-ray-absorbing liquid deposited. For this lung, there was a good agreement among the two *ex vivo* imaging modalities (CT rendering and epifluorescence whole lung imaging depicted in (Fig. 2 (c) and (d), respectively) and the *in vivo* observation that the liquid was not homogeneously delivered over the whole lung, but preferentially in the right lung of the mouse (seen on the left side of the image). In Fig. 2 (e), a photograph of a typical *ex vivo* lung after drying is shown. The entire time sequence of images for this delivery (Video 3), as well as the rendering (Video 4), can be found in the supplementary material.

Figure 4: In the pseudo-colored images (a) - (c) the analyzed region of interests are highlighted. In (d) - (f) the corresponding plots are displayed. The subjects displayed in (a) and (b) received a $50\,\mu l$ delivery, and the one in (c) received a $4\,\mu l$ delivery. The plots describe how long it takes for the liquid to arrive in different parts of the lung. Time = 0 s was set as the time when the liquid first appeared in the field of view. The end of delivery after 12.5 s ((d) and (e)) and 5 s in (f) is indicated by the dashed vertical line. Scale bar: 2 mm

Fig. 3 (a) and (b) illustrate the ability of this *in vivo* method to capture not only the spatial distribution of treatment, after pulmonary delivery of $50\,\mu l$ of liquid via instillation, but also the temporal evolution of the liquid/drug delivery process. In (a) the liquid was primarily delivered into one lobe (again the post-caval lobe), resulting in a highly-localized deposition profile of the liquid, while in (b) a more uniform distribution throughout the entire lung (global distribution) is seen (the time-sequence movies can be found in the supplementary material, Video 5 and 6). This allows identification of temporal features of the instillation process. For instance in (b) we can

clearly observe how the liquid enters and gradually accumulates in both the left and right bronchus. After a sufficient amount of liquid has been accumulated in the bronchi (here: 16.32 s) the liquid is distributed rapidly (within a few seconds) throughout the entire lung reaching even the most peripheral (alveolar) region. Considering that the entire 50 µl of liquid was delivered within 12.5 s the significant redistribution of the drug is likely due to gravitational forces (the mouse is located in an upright position) as well as breathing activity.

In a second type of experiment we performed imaging while the mouse was breathing freely, and delivered a reduced volume of Liquid I. As shown in Fig. 3 (c), the small volume of liquid distributed mostly in one lobe. Nevertheless, we were still able to detect a signal and track this very small volume during the pulmonary application process (time-sequence movie can be found in the supplementary material, Video 7). It is noteworthy that, as in Fig. 3 (b), there was a significant redistribution of the instilled liquid even after all of the liquid had been applied (at 5.0 s). Again, liquid from the primary bronchus was transported into more peripheral regions of the (right) lung, likely due to gravity and respiration.

Each lung shown in Fig. 3 was analyzed regionally by selecting one or more regions of interest, as displayed in Fig. 4 (a)-(c). The percentage of deposited treatment volume was calculated for each image in the sequence, as explained in the method section. Fig. 4 (d) - (f) not only confirms the qualitative trends observed in Fig. 4 (a) - (c) in a more quantitative way, it also provides highly temporally resolved information on the liquid transport rates (slopes of signals) and the regional redistribution of the delivered liquid. Fig. 4 (d) shows that the liquid rapidly reached the lungs within the delivery period (12.5 s), starting at a relatively low rate that abruptly increased at about 10 s. Once the maximum dose was reached the signals from both ROIs remained at a relatively constant level, except for ROI 1, which gradually decreased, starting from around 100 s. Fig. 4 (e) shows different delivery kinetics to Fig. 4 (d). After an initial burst of deposition almost no liquid was appearing until another strong burst at about 25 s, which was about 12 s after the instillation process ended. We attribute this to transport of residual liquid deposited in the intubation cannula and trachea, which were out of the field of view. After 25 s the fluid redistributed from the central region (ROI 1) to the more peripheral regions of the lung (ROI 2 and ROI 5) for the entire observation period (300 s). This effect was not evident on the right side of the image (left half of the lung). The delivery kinetics observed for the spontaneously breathing mouse that received only 4 µl of liquid (Fig. 4 (f)) were very similar to the kinetics depicted in

Fig. 4 (d). Hence, observed delivery kinetics are likely not dependent on the type of breathing or the amount of delivered liquid.

After the *in vivo* experiment, fluorescence images of some dried excised lungs were acquired, see Fig. 3 (d)-(f), which do not always completely agree with the *in vivo* observed distribution. We hypothesise that this could be because the dye distribution in the lung changes during the drying procedure, and the fluorescence measurements are most sensitive to the liquid deposited close to the surface of the lung (due to light attenuation by the tissue).

3.2. Inhalation

Inhalation of aerosolized drugs is widely used for treatment of chronic lung diseases such as asthma, COPD and cystic fibrosis. At the moment, only the European Synchrotron Radiation Facility ID17 beamline is capable of fast dual-energy synchrotron radiation CT imaging, able to acquire *in vivo* images showing regional deposition of specific elements (i.e. iodine, xenon), lung morphology and regional ventilation at the same time, using K-edge subtraction CT imaging [38]. To the best of our knowledge, the results presented here are the first proof-of-principle study to image a dynamic inhalation process *in vivo* with x-rays in a laboratory environment. Aerosol inhalation is more challenging to image than instillation, because the volume of inhaled liquid delivered to the lung is relatively small (only about $<$ 10 % of nominal dose is actually deposited in the lung) and distributed uniformly throughout the lung as shown by Yang et al. [63]. Therefore, we performed preparatory experiments to maximize the lung-delivered aerosol volume. First, the operating parameters of the small animal ventilator were optimized for aerosol delivery to the intubation cannula (mouse) by measuring the inhaled aerosol dose with an *in vitro* gravimetric method [64]. This resulted in selection of a slow deep inhalation pattern (80 breath/min; tidal volume 600 µl for 20 g mouse) with a 204 ms breath hold for imaging at the end of the 298 ms inspiratory phase. For a nebulizer active-time of 40 ms per breath, 150 µl of liquid was nebulized within 660 s.

Figure 5: A time sequence of mouse lung images, collected while inhaling a nebulized liquid. Bubbles (yellow arrows) are observed in the airways after several minutes of inhalation, and remain for several seconds before they burst. Note that the defined features along the pleura (boundary of the lung) and the ribs are a result of motion between frames. Time 0 s was set to the

start of inhalation. Scale bar: 2 mm.

As shown in Fig. 5, using pseudo-colored images we were able to visualize the inhalation of the iodine-based liquid, and determine if the liquid deposited on the airway surface. The strongest contrast was found when the inhaled liquid formed bubbles, with radii ranging from $300\,\mu m$ to 1.6 mm in diameter (in all images of Fig. 5; indicated by yellow arrows). Bubble formation was not observed for all inhalation experiments, but in the one animal in which we saw this, the bubbles began to appear at around 200 s into the imaging sequence, and remained for a few seconds in the primary bronchi before they burst and distributed into the lung. A movie of the entire time sequence can be found in the supplementary material (Video 8). The images are obtained during the first run of nebulization. A second run with $150\,\mu l$ nebulized liquid was preformed on this mouse. In total $300\,\mu l$ of liquid was nebulized. Due to aerosol deposition within the ventilator circuit, and partial aerosol deposition in the lung, only $24\,\mu l$ of liquid was deposited in the lung (as determined by epifluorescence imaging on 1 mm lung slices).

This amount was delivered within 22 min, i.e. the liquid delivery rate of $0.018\,\mu l/s$ which is much slower than for the $4\,\mu l$ instillation ($0.8\,\mu l/s$) and the delivered aerosol volume was more than 5-fold the $4\,\mu l$ applied via instillation. In contrast to the clearly distinguishable signal for the $4\,\mu l$ instillation (Fig 3 (c), 4 (c) and (f)), detecting a signal from the aerosolized fluid was much more challenging following inhalation, due to the extremely uniform distribution of inhaled aerosol throughout the entire lung that resulted in a low signal-to-noise ratio [63]. Consequently, only localized features such as bubbles or some minor hot spots could be determined (yellow arrows in Fig. 5). Moreover, sometimes during the inhalation period some signal was detected near regions of strong contrast change such as at the ribs or near the pleura (rim) of the lung. These features are an artifact that arise when local motion of anatomical features cannot be separated from the motion of the treatment.

4. Discussion

For pre-clinical and clinical lung disease research, accurate determination of dose and regional distribution of pulmonary applied substances is essential for efficacy and toxicity analysis. Lung diseases can be localized to the bronchial (e.g. asthma, COPD, bronchitis, or cystic fibrosis),

alveolar (e.g. pneumonia, tuberculosis, pulmonary edema) or interstitial (e.g. interstitial pulmonary fibrosis) regions. For this reason, targeted delivery of treatments to the lungs of animals and humans is an area of active research (e.g. [65]). The scarcity of *in vivo* drug dosimetry methods was the motivation for using dynamic phase-contrast imaging to monitor deposition in the lungs of mice. The aim was to visualize and quantify the regional lung deposition of instilled or inhaled liquid in living animals with a high temporal resolution. We demonstrated that for the instillation process this method is capable of visualizing and quantifying the dynamics of fluid volumes as small as $4\,\mu l$. However, visualization of aerosol inhalation is more challenging, since the local contrast changes are much weaker due to the extremely uniform distribution of the liquid throughout the lung and smaller local volumes.

The time-sequence imaging of treatment delivery in live mice revealed several interesting results. It is well known that aerosol inhalation provides a more uniform pulmonary drug distribution than instillation [63], but considering the relatively crude means of application (bulk liquid - not aerosol - is squirted into the lungs) the relatively uniform pulmonary distributions of instilled substances was quite surprising. Our time-sequences indicated that this is likely a result of secondary aerosolization. As seen in the supplementary movies, the liquid first collects in the upper airways relatively close to the entrance points (e.g. the tip of intubation cannula in the trachea). Initially some liquid flows slowly down the airway tree. Once enough liquid has accumulated to block the airway the liquid gets sucked into the lung with the next breath resulting in secondary aerosolization and thus relatively uniform redistribution of the liquid throughout the lung. Since this low energy process of aerosol formation is similar to low energy droplet formation at the tip of breaking waves, one would expect a similar droplet size distribution with droplets much larger than $10\,\mu m$ in diameter [66]. Droplets of this size will not be able to reach the alveolar region of the murine lung [6], which is consistent with the central and patchy deposition of intratracheally instilled liquids as observed by Lin et al. [63]. However, our *in vivo* imaging revealed that gradual redistribution of instilled liquid (iodine) from the central to the peripheral region occurs even for minutes after the instillation process has ended (Fig. 4 (b) and (e)). As a caveat, we note that we delivered $4\,\mu l$ or $50\,\mu l$ liquid within 5 and 12.5 s, respectively, while standard intratracheal instillation protocols squirt the liquid into the lung within < 1 s. While this is expected to change the time-scale of events, the general mechanism of secondary aerosol formation is likely to be relevant for both instillation protocols. The former is consistent with hot

spots reported in our previous study using whole lung *ex vivo* light sheet fluorescence microscopy, which were attributed to occasional redistribution of liquid collected in the trachea [63]. This could also be the reason for the observed bubble formation during inhalation of aerosolized treatment. Since the applied liquid did not contain any detergent or proteins, direct bubble formation was unlikely. On the other hand, if aerosolized liquid was first deposited on the lung epithelium and then redistributed in a similar fashion to that described for instillation, it may have contained proteins present in the lung lining fluid, which may result in bubble formation. This behaviour was observed in just one mouse, and occurred at the timepoint when $\sim 3.6\,\mu l$ of liquid had reached the lung. Again as a caveat, we note that ventilator assisted aerosol delivery is typically performed with the subject positioned horizontally and not vertically as done here. Nevertheless, the possibility of occasional bubble formation may also occur for ventilator-assisted aerosol inhalation for horizontally-oriented subjects.

A final interesting observation from the live imaging was that the liquid continued to gradually move from the central to the peripheral regions of the lung for some minutes after the instillation process had been terminated. This is important when considering the timing of delivery in non-recovery laboratory experiments. A future study could look at even longer timescales to determine if the liquid is taken up by the bloodstream, possibly reducing the contrast observed within the lung.

Subsequent multi-modal *ex vivo* imaging can provide complementary information. *Ex vivo* CT scans of the dried lungs provided more refined three dimensional spatial localisation of the iodine (drug) signal and co-registration of lung morphology in a non-destructive way. On the other hand, whole (dried) lung epifluorescence imaging provided two dimensional and relatively superficial information on lung shape and dye (drug) distribution. However, after slicing the dried lung, dosimetry and three dimensional resolved drug distribution can be obtained with quantitative fluorescence spectroscopy [62]. One should also note that the liquid distribution might change during the lung drying process. For example, one interesting difference that we noted was that the whole lung fluorescence images (e.g. Fig. 3 (e)) do not show strong signals when the treatment is not close to the surface (c.f. Fig. 3 (b)). This was due to the relatively high tissue attenuation of the fluorescence which limits the penetration depth to about 1 mm even in the near infrared regime as used here [62]. One should also recognize that CT scanning and fluorescence imaging rely on different agents (iodine and Sky Blue, respectively) for detection. Thus different biokinetics of

these agents may also result in differences in the observed *ex vivo* images. Nevertheless, *ex vivo* imaging may provide complementary information (e.g. more resolved 3D information and quantitative dosimetry) to *in vivo* imaging.

We have shown, through comparison with CT and fluorescence data, that with the instillation visualization techniques presented here we are able to reliably predict the distribution of the liquid, which can help to determine the influence of different delivery methods on the resulting distribution (e.g. the speed of delivery, the depth of the intubation). In future studies we could also examine the influence of lung disease on drug distribution in pre-clinical models. An advantage of the approach described here is that dynamic and anatomic information are collected simultaneously. When using nuclear imaging methods, no anatomic information is available, which often means that additional x-ray images and registration processes are necessary. In addition, compared to nuclear imaging methods the increased spatial resolution provides more detailed information on distribution within a specific lobe. This setup can also be further improved by using a syringe pump that can deliver more quickly, so that deliveries within 1 s can be performed. With further improvement of the inverse Compton x-ray source the x-ray flux will increase, allowing for faster imaging and therefore an even higher temporal resolution.

In this study *in vivo* x-ray imaging has provided insight into the temporal evolution of pulmonary drug delivery and secondary redistribution of the drug within the lung, both during and after the delivery. These findings can contribute to optimized future pulmonary drug delivery studies. While volumes of only a few μl were visible following instillation of liquids, for uniformly-distributed inhaled nebulized liquid throughout the entire lung, our x-ray imaging setup is not sensitive enough for reliable drug localization. While the present study focused on lung, the method presented here could also be used for transport and retention studies on other organs or tissue sections. But of course similar validation studies as performed here would be recommended. We envision that the techniques presented here have a range of applications in pre-clinical animal model research (e.g. drug delivery, device testing, and treatment assessment), rather than clinical settings, due to the x-ray source properties (beam size, x-ray energy) and relatively high deposited radiation dose because of the desired high temporal and spatial resolution. As an application of this technique we identified secondary aerosol formation as a mechanism for distribution of intratracheally instilled bulk liquids throughout the entire lungs of animal models. Time-resolved imaging of the aerosol delivery process is limited due to the small local changes in light

attenuation as a result of the extremely uniform pulmonary distribution of inhaled aerosol. Possible future directions include combining this assessment with bronchoscopic dose delivery (including a small camera in the airways [65]) to target a particular lobe and verify that the treatment deposits correctly in that location.

Appendix A. Movies

Where appropriate, the sequence is accelerated to 5 fps and compressed to reduce file size.

Video 1: Treatment delivery sequence for Fig. 1

Temporal profile of liquid (iodine) distribution in the lung during and after instillation of 50 µl into the lung.

Video 2: Subtracted images without treatment delivery

The validity of the assumption that the subtracted pre-delivery image remains constant during the entire imaging period was confirmed by obtaining no significant difference signal for blank lungs.
‘

Video 3: Treatment delivery sequence for Fig. 2

Temporal profile of liquid (iodine) distribution in the lung during and after instillation of 50 µl instillation, resulting in a local distribution.

Video 4: Rendering of Fig. 2 (c)

Visualization of the whole lung CT rendering from Fig. 2 (c).

Video 5: Treatment delivery sequence for Fig. 3 (a)

Temporal profile of liquid (iodine) distribution in the lung during and after instillation of 50 µl instillation, resulting in a local distribution.

Video 6: Treatment delivery sequence for 3 (b)

Temporal profile of liquid (iodine) distribution in the lung during and after instillation of 50 µl instillation into the lung, resulting in a global distribution.

Video 7: Treatment delivery sequence for 3 (c)

Temporal profile of liquid (iodine) distribution in the lung during and after instillation of 4 µl instillation into the lung, resulting a local distribution.

Video 8: Inhalation sequence of Fig. 5

Temporal profile of liquid (iodine) distribution in the lung during and after instillation of the inhalation process.

References

[1] S. Dubsky, A. Fouras, Imaging regional lung function: A critical tool for developing inhaled antimicrobial therapies, Advanced Drug Delivery Reviews 85 (2015) 100–109.

[2] H. A. Tiddens, A. C. Bos, J. W. Mouton, S. Devadason, H. M. Janssens, Inhaled antibiotics: Dry or wet?, European Respiratory Journal 44 (5) (2014) 1308–1318.

[3] O. Schmid, F. Cassee, On the pivotal role of dose for particle toxicology and risk assessment: Exposure is a poor surrogate for delivered dose, Part Fibre Toxicol. 14 (52).

[4] . Darquenne, J. Fleming, I. Katz, A. Martin, J. Schroeter, O. Usmani, J. Venegas, O. Schmid, The gap between science and clinical efficacy: Physiology, imaging and modeling of aerosols in the lung, J. Aerosol Med. Pulm. Drug Deliv. 29 (2) (2016) 107–126.

[5] P. Cmielewski, N. Farrow, S. Devereux, D. Parsons, P. Cmielewski, N. Farrow, S. Devereux, D. Parsons, P. Cmielewski, Gene therapy for Cystic Fibrosis : Improved delivery techniques and conditioning with lysophosphatidylcholine enhance lentiviral gene transfer in mouse lung airways, Experimental Lung Research 43 (9) (2017) 426–433.

[6] W. Hofmann, Modelling inhaled particle deposition in the human lung-A review, Journal of Aerosol Science 10 (42) (2011) 693–724.

[7] F. Lizal, J. Jedelsky, K. Morgan, K. Bauer, J. Llop, U. Cossio, S. Kassinos, S. Verbanck, J. Ruiz-Cabello, A. Santos, et al., Experimental methods for flow and aerosol measurements in human airways and their replicas, European Journal of Pharmaceutical Sciences 113 (2018)

95–131.

[8] E. M. Eslick, J. Kipritidis, D. Gradinscak, M. J. Stevens, D. L. Bailey, B. Harris, J. T. Booth, P. J. Keall, CT ventilation imaging derived from breath hold CT exhibits good regional accuracy with Galligas PET, Radiother. Oncol. 127 (2) (2018) 267–273.

[9] C. Hoyer, N. Gass, W. Weber-Fahr, A. Sartorius, Advantages and challenges of small animal magnetic resonance imaging as a translational tool, Neuropsychobiology 69 (4) (2014) 187–201.

[10] H. Benveniste, S. Blackband, MR microscopy and high resolution small animal MRI: Applications in neuroscience research, Progress in Neurobiology 67 (5) (2002) 393–420.

[11] B. Driehuys, J. Nouls, A. Badea, E. Bucholz, K. Ghaghada, A. Petiet, L. W. Hedlund, Small Animal Imaging with Magnetic Resonance Microscopy, ILAR Journal 49 (1) (2008) 35–53.

[12] D. P. Schuster, A. Kovacs, J. Garbow, D. Piwnica-Worms, Translational Review Recent Advances in Imaging the Lungs of Intact Small Animals, American Journal of Respiratory Cell and Molecular Biology 30 (2004) 129–138.

[13] J. Conway, Lung imaging - Two dimensional gamma scintigraphy, SPECT, CT and PET, Advanced Drug Delivery Reviews 64 (4) (2012) 357–368.

[14] A. Fouras, M. J. Kitchen, S. Dubsky, R. A. Lewis, S. B. Hooper, K. Hourigan, The past, present, and future of x-ray technology for in vivo imaging of function and form, Journal of Applied Physics 105 (10) (2009) 102009.

[15] A. Momose, S. Kawamoto, I. Koyama, Y. Hamaishi, K. Takai, Y. Suzuki, Demonstration of x-ray Talbot interferometry, Japanese Journal of Applied Physics 42 (7 B) (2003) L866–L868.

[16] T. Weitkamp, A. Diaz, C. David, F. Pfeiffer, M. Stampanoni, P. Cloetens, E. Ziegler, X-ray phase imaging with a grating interferometer., Optics express 13 (16) (2005) 6296–6304.

[17] P. Cloetens, R. Barrett, J. Baruchel, J.-P. Guigay, M. Schlenker, Phase objects in synchrotron radiation hard x-ray imaging, J. Appl. Phys. 29 (1996) 133–146.

[18] A. Snigirev, I. Snigireva, V. Kohn, S. Kuznetsov, I. Schelokov, On the possibilities of x-ray phase contrast microimaging by coherent high-energy synchrotron radiation, Review of Scientific Instruments 66 (12) (1995) 5486.

[19] F. Pfeiffer, T. Weitkamp, O. Bunk, C. David, Phase retrieval and differential phase-contrast imaging with low-brilliance X-ray sources, Nat. Phys. 2 (2006) 258–261.

[20] R. Gradl, K. S. Morgan, M. Dierolf, C. Jud, L. Hehn, B. Günther, W. Möller, D. Kutschke, L. Yang, T. Stoeger, D. Pfeiffer, B. Gleich, K. Achterhold, O. Schmid, F. Pfeiffer, Dynamic in vivo chest x-ray dark-field imaging in mice, IEEE Trans. Med. Imaging 38 (2) (2019) 649–656.

[21] S. K. Lynch, V. Pai, J. Auxier, A. F. Stein, E. E. Bennett, C. K. Kemble, X. Xiao, W.-K. Lee, N. Y. Morgan, H. H. Wen, Interpretation of dark-field contrast and particle-size selectivity in grating interferometers, Applied Optics 50 (22) (2011) 4310–4319.

[22] P. Modregger, M. Kagias, S. C. Irvine, R. Brönnimann, K. Jefimovs, M. Endrizzi, A. Olivo, Interpretation and Utility of the Moments of Small-Angle X-Ray Scattering Distributions, Physical Review Letters 118 (26) (2017) 265501.

[23] A. Yaroshenko, K. Hellbach, A. Ö. Yildirim, T. M. Conlon, I. E. Fernandez, M. Bech, A. Velroyen, F. G. Meinel, S. Auweter, M. Reiser, O. Eickelberg, F. Pfeiffer, Improved In vivo Assessment of Pulmonary Fibrosis in Mice using X-Ray Dark-Field Radiography, Scientific Reports 5 (2015) 17492.

[24] S. Schleede, M. Bech, K. Achterhold, G. Potdevin, M. Gifford, R. Loewen, C. Limborg, R. Ruth, F. Pfeiffer, Multimodal hard X-ray imaging of a mammography phantom at a compact synchrotron light source, J. Synchrotron Rad. 19 (2012) 525–529.

[25] F. G. Meinel, F. Schwab, S. Schleede, M. Bech, J. Herzen, K. Achterhold, S. Auweter, F. Bamberg, A. Ö. Yildirim, A. Bohla, O. Eickelberg, R. Loewen, M. Gifford, R. Ruth, M. F. Reiser, F. Pfeiffer, K. Nikolaou, Diagnosing and Mapping Pulmonary Emphysema on X-Ray Projection Images: Incremental Value of Grating-Based X-Ray Dark-Field Imaging, PLoS ONE (2013) e59526.

[26] K. Scherer, A. Yaroshenko, D. A. Bölükbas, L. B. Gromann, K. Hellbach, F. G. Meinel, M. Braunagel, J. Von Berg, O. Eickelberg, M. F. Reiser, F. Pfeiffer, S. Meiners, J. Herzen, X-ray Dark-field Radiography - In-Vivo Diagnosis of Lung Cancer in Mice, Scientific Reports 7 (1) (2017) 402.

[27] F. Schwab, S. Schleede, D. Hahn, M. Bech, J. Herzen, S. Auweter, F. Bamberg, K. Achterhold, A. Ö. Yildirim, A. Bohla, O. Eickelberg, R. Loewen, M. Gifford, R. Ruth, M. F. Reiser, K. Nikolaou, F. Pfeiffer, F. G. Meinel, Comparison of contrast-to-noise ratios of transmission and dark-field signal in grating-based X-ray imaging for healthy murine lung tissue, Zeitschrift fur Medizinische Physik (2013) 236–242.

[28] L. B. Gromann, F. De Marco, K. Willer, P. B. Noël, K. Scherer, B. Renger, B. Gleich, K. Achterhold, A. A. Fingerle, D. Muenzel, S. Auweter, K. Hellbach, M. Reiser, A. Baehr, M. Dmochewitz, T. J. Schroeter, F. J. Koch, P. Meyer, D. Kunka, J. Mohr, A. Yaroshenko, H. I. Maack, T. Pralow, H. Van Der Heijden, R. Proksa, T. Koehler, N. Wieberneit, K. Rindt, E. J. Rummeny, F. Pfeiffer, J. Herzen, In-vivo X-ray Dark-Field Chest Radiography of a Pig, Scientific Reports 7 (1) (2017) 4807.

[29] K. Willer, A. A. Fingerle, L. B. Gromann, F. De Marco, J. Herzen, K. Achterhold, B. Gleich, D. Muenzel, K. Scherer, M. Renz, B. Renger, F. Kopp, F. Kriner, F. Fischer, C. Braun, S. Auweter, K. Hellbach, M. F. Reiser, T. Schroeter, J. Mohr, A. Yaroshenko, H.-I. Maack, T. Pralow, H. van der Heijden, R. Proksa, T. Koehler, N. Wieberneit, K. Rindt, E. J. Rummeny, F. Pfeiffer, P. B. Noël, X-ray dark-field imaging of the human lunga feasibility study on a deceased body, PLoS ONE 13 (9) (2018) e0204565.

[30] S. W. Wilkins, T. E. Gureyev, D. Gao, A. Pogany, A. W. Stevenson, Phase-contrast imaging using polychromatic hard X-rays, Nature 384 (1996) 335–338.

[31] P. Bidola, K. Morgan, M. Willner, A. Fehringer, S. Allner, F. Prade, F. Pfeiffer, K. Achterhold, Application of sensitive, high-resolution imaging at a commercial lab-based X-ray micro-CT system using propagation-based phase retrieval, Journal of Microscopy 266 (2) (2017) 211–220.

[32] S. Bayat, G. Le Duc, L. Porra, G. Berruyer, C. Nemoz, S. Monfraix, S. Fiedler, W. Thomlinson, P. Suortti, C.-G. Standertskjöld-Nordenstam, et al., Quantitative functional lung imaging with synchrotron radiation using inhaled xenon as contrast agent, Physics in Medicine & Biology 46 (12) (2001) 3287.

[33] R. Lewis, N. Yagi, M. Kitchen, M. Morgan, D. Paganin, K. Siu, K. Pavlov, I. Williams, K. Uesugi, M. Wallace, et al., Dynamic imaging of the lungs using x-ray phase contrast, Physics in Medicine & Biology 50 (21) (2005) 5031.

[34] S. B. Hooper, M. J. Kitchen, M. J. Wallace, N. Yagi, K. Uesugi, M. J. Morgan, C. Hall, K. K. W. Siu, I. M. Williams, M. Siew, S. C. Irvine, K. Pavlov, R. A. Lewis, Imaging lung aeration and lung liquid clearance at birth, The FASEB Journal 21 (12) (2007) 3329–3337.

[35] M. J. Kitchen, D. Paganin, R. A. Lewis, N. Yagi, K. Uesugi, S. T. Mudie, On the origin of speckle in x-ray phase contrast images of lung tissue, Physics in Medicine and Biology 49 (18) (2004) 4335.

[36] M. Donnelley, K. K. W. Siu, R. A. Jamison, D. W. Parsons, Synchrotron phase-contrast X-ray imaging reveals fluid dosing dynamics for gene transfer into mouse airways, Gene Therapy 19 (1) (2011) 8–14.

[37] K. S. Morgan, M. Donnelley, N. Farrow, A. Fouras, N. Yagi, Y. Suzuki, A. Takeuchi, K. Uesugi, R. Boucher, K. Siu, D. Parsons, In Vivo X-Ray Imaging Reveals Improved Airway Surface Hydration after a Therapy Designed for Cystic Fibrosis, American Journal of Respiratory and Critical Care Medicine 190 (4) (2014) 469–472.

[38] L. Porra, L. Dégrugilliers, L. Broche, G. Albu, S. Strengell, H. Suhonen, G. H. Fodor, F. Peták, P. Suortti, W. Habre, A. R. Sovijärvi, S. Bayat, Quantitative Imaging of Regional Aerosol Deposition, Lung Ventilation and Morphology by Synchrotron Radiation CT, Scientific Reports 8 (1) (2018) 3519.

[39] O. Hemberg, M. Otendal, H. M. Hertz, Liquid-metal-jet anode electron-impact x-ray source, Applied Physics Letters 83 (7) (2003) 1483–1485.

[40] Z. Chi, L. Yan, Y. Du, Z. Zhang, W. Huang, H. Chen, C. Tang, Recent progress of phase-contrast imaging at Tsinghua Thomson-scattering X-ray source, Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms (2017) 364–369.

[41] R. Gradl, M. Dierolf, K. Achterhold, B. Günther, B. Gleich, F. Pfeiffer, K. Morgan, Propagation-based X-ray phase-contrast Imaging at a Compact Light Source, Scientific Reports 7 (2017) 4908.

[42] J. Wenz, S. Schleede, K. Khrennikov, M. Bech, P. Thibault, M. Heigoldt, F. Pfeiffer, S. Karsch, Quantitative X-ray phase-contrast microtomography from a compact laser-driven betatron source., Nature Communications (2015) 7568.

[43] J. R. Loewen, A compact light source: Design and technical feasibility study of a LASER-electron storage ring X-ray source, Ph.D. thesis, Stanford University, 2003.

[44] H. Ikeura-Sekiguchi, R. Kuroda, M. Yasumoto, H. Toyokawa, M. Koike, K. Yamada, F. Sakai, K. Mori, K. Maruyama, H. Oka, T. Kimata, In-line phase-contrast imaging of a biological specimen using a compact laser-Compton scattering-based x-ray source, Applied Physics Letters 92 (13) (2008) 131107.

[45] D. H. Larsson, W. Vågberg, A. Yaroshenko, A. Ö. Yildrim, H. M. Hertz, High-resolution short-exposure small-exposure small-animal laboratory x-ray phase-contrast tomography,

Scientific Reports 6 (2016 a) 39074.

[46] M. Preissner, R. Murrie, I. Pinar, F. Werdiger, R. Carnibella, G. Zosky, A. Fouras, S. Dubsky, High resolution propagation-based imaging system for in vivo dynamic computed tomography of lungs in small animals, Physics in Medicine & Biology 63 (8) (2018) 08NT03.

[47] E. H. Kim, M. Preissner, R. Carnibella, C. R. Samarage, E. Bennett, A. Fouras, G. R. Zosky, H. D. Jones, Novel Analysis of 4DCT Imaging Quantifies Progressive Increases in Anatomic Dead Space During Mechanical Ventilation in Mice, Journal of Applied Physiology (2017) jap.00903.2016.

[48] R. Gradl, M. Dierolf, B. Günther, L. Hehn, W. Möller, D. Kutschke, L. Yang, M. Donnelley, R. Murrie, A. Erl, et al., In vivo Dynamic Phase-Contrast X-ray Imaging using a Compact Light Source, Scientific Reports 8 (1) (2018) 6788.

[49] E. Eggl, M. Dierolf, K. Achterhold, C. Jud, B. Günther, E. Braig, B. Gleich, F. Pfeiffer, The Munich Compact Light Source: initial performance measures, J. Synchrotron Rad. 23 (5) (2016) 1137–1142.

[50] K. Achterhold, M. Bech, S. Schleede, G. Potdevin, R. Ruth, R. Loewen, F. Pfeiffer, Monochromatic computed tomography with a compact laser-driven X-ray source, Scientific Reports 3 (2013) 1313.

[51] Lyncean Technologies Inc., The Compact Light Source, URL: http://www.lynceantech.com/, [Online; accessed 2019-02-06], 2019.

[52] A. F. Leong, G. A. Buckley, D. M. Paganin, S. B. Hooper, M. J. Wallace, M. J. Kitchen, Real-time measurement of alveolar size and population using phase contrast x-ray imaging, Biomed. Opt. Express 5 (11) (2014) 4024.

[53] M. J. Kitchen, G. A. Buckley, A. F. Leong, R. P. Carnibella, A. Fouras, M. J. Wallace, S. B. Hooper, X-ray specks: Low dose in vivo imaging of lung structure and function, Phys. Med. Biol. 60 (18) (2015) 7259–7276.

[54] D. H. Larsson, W. Vågberg, A. Yaroshenko, A. Ö. Yildirim, H. M. Hertz, High-resolution short-exposure small-animal laboratory x-ray phase-contrast tomography, Sci. Rep. 6 (2016 b) 39074.

[55] T. Rodt, M. Luepke, C. Boehm, C. Von Falck, G. Stamm, J. R. Borlak, H. Seifert, M. Galanski, Phantom and cadaver measurements of dose and dose distribution in micro-CT of

the chest in mice, Acta radiol. 52 (1) (2011) 75–80.

[56] R. H. Brown, D. M. Walters, R. S. Greenberg, W. Mitzner, A method of endotracheal intubation and pulmonary functional assessment for repeated studies in mice, Journal of Applied Physiology 87 (6) (1999) 2362–2365.

[57] M. Donnelley, K. S. Morgan, K. K. Siu, D. W. Parsons, Variability of In Vivo Fluid Dose Distribution in Mouse Airways Is Visualized by High-Speed Synchrotron X-Ray Imaging, Journal of Aerosol Medicine and Pulmonary Drug Delivery 26 (5) (2013) 307.

[58] M. Donnelley, K. S. Morgan, R. Gradl, M. Klein, D. Hausermann, C. Hall, A. Maksimenko, D. W. Parsons, Live-pig-airway surface imaging and whole-pig CT at the Australian Synchrotron Imaging and Medical, J. Synchrotron Radiat. 26 (2019) 175–183.

[59] E. Jones, T. Oliphant, P. Peterson, et al., SciPy: Open source scientific tools for Python, URL: `http://www.scipy.org/`, [Online; accessed 2019-02-06], 2019.

[60] J. Döpfert, Roipoly, URL: `https://github.com/jdoepfert/roipoly.py`, [Online; accessed 2019-02-06], 2019.

[61] M. L. Harbison, J. D. Brain, Effects of Exercise on Particle Deposition in Syrian Golden Hamsters, The American Review of Respiratory Disease 128 (5) (1983) 904–8.

[62] N. Barapatre, P. Symvoulidis, W. Möller, F. Prade, N. Deliolanis, S. Hertel, G. Winter, A. Ö. Yildirim, T. Stoeger, O. Eickelberg, V. Ntziachristos, O. Schmid, Quantitative detection of drug dose and spatial distribution in the lung revealed by Cryoslicing Imaging, Journal of Pharmaceutical and Biomedical Analysis 102 (2015) 129–136.

[63] L. Yang, A. Feuchtinger, W. Moeller, Y. Ding, D. Kutschke, G. Möller, J. C. Schittny, G. Burgstaller, W. Hofmann, T. Stoeger, D. Razansky, A. Walch, O. Schmid, Three-Dimensional Quantitative Co-Mapping of Pulmonary Morphology and Nanoparticle Distribution with Cellular Resolution in Non-Dissected Murine Lungs, ACS Nano .

[64] A. Robichaud, L. Fereydoonzad, T. Schuessler, Delivered dose estimate to standardize airway hyperresponsiveness assessment in mice, American Journal of Physiology - Lung Cellular and Molecular Physiology 8 (308) (2015) L837–L846.

[65] C. McIntyre, M. Donnelley, N. Rout-Pitt, D. Parsons, Lobe-specific gene vector delivery to rat lungs using a miniature bronchoscope, Human gene therapy methods 29 (5) (2018) 228–235.

[66] W. C. Hinds, Aerosol technology: Properties, behavior, and measurement of aiborne

particles, New York: Wiley, 1999.

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Highlights:

- *In vivo* visualization of pulmonary drug distribution, during and post delivery
- High temporal and spatial resolution imaging from an inverse Compton x-ray source
- Anatomical information and treatment distribution collected simultaneously
- X-ray images improve understanding of instillation and nebulized drug delivery