Multimodal Precision Imaging of Pulmonary Nanoparticle Delivery in Mice: Dynamics of Application, Spatial Distribution, and Dosimetry

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Targeted delivery of nanomedicine/nanoparticles (NM/NPs) to the site of disease (e.g., the tumor or lung injury) is of vital importance for improved therapeutic efficacy. Multimodal imaging platforms provide powerful tools for monitoring delivery and tissue distribution of drugs and NM/NPs. This study introduces a preclinical imaging platform combining X-ray (two modes) and fluorescence imaging (three modes) techniques for time-resolved in vivo and spatially resolved ex vivo visualization of mouse lungs during pulmonary NP delivery. Liquid mixtures of iodine (contrast agent for X-ray) and/or (nano)particles (X-ray absorbing and/or fluorescent) are delivered to different regions of the lung via intratracheal instillation, nasal aspiration, and ventilator-assisted aerosol inhalation. It is demonstrated that in vivo propagation-based phase-contrast X-ray imaging elucidates the dynamic process of pulmonary NP delivery, while ex vivo fluorescence imaging (e.g., tissue-cleared light sheet fluorescence microscopy) reveals the quantitative 3D drug/particle distribution throughout the entire lung with cellular resolution. The novel and complementary information from this imaging platform unveils the dynamics and mechanisms of pulmonary NM/NP delivery and deposition for each of the delivery routes, which provides guidance on optimizing pulmonary delivery techniques and novel-designed NM for targeting and efficacy.

1. Introduction

Lung diseases are among the leading causes of deaths worldwide. According to the World Health Organization[1] the chronic obstructive pulmonary disease (COPD) and lung cancer have even been rising from the fourth and ninth in 2000 to the third and sixth of most deadly disease in 2016, respectively. For treatment of lung diseases, direct application of the drug to the lung via aerosol inhalation is widely used due to its high therapeutic index, i.e., high efficacy at the site of disease and low side effects in secondary organs. Currently, inhalation therapy is a cornerstone for the treatment of pulmonary infections, asthma, COPD, and cystic fibrosis, but also treatment of lung cancer or even nonpulmonary disease such as diabetes is within reach.[2,3] Also nanotechnology enabled drugs (nanomedicines (NMs)) offer new diagnostic and therapeutic options for cancer and other diseases leveraging their unique features for controlled release, reduced drug toxicity, prolonged...
residence, and/or targeted delivery.[4–6] Consequently, numerous NM formulations are already in clinical use or are currently undergoing clinical trials.[7,8] In spite of this success a recent study reported that only about 0.7% of the applied dose of intravenously administered nanoparticles (NPs) accumulates at the tumor sites in animal models,[9] suggesting that targeting delivery of NM is still a major subject of both preclinical and clinical studies.

To better understand and optimize drug/NM delivery to the sites of disease within the lung, it is of central importance to quantitatively monitor the dynamic process of pulmonary drug delivery in the context of deposited dose, initial biodistribution, site-specific localization, biokinetics, and therapeutic efficacy (bioactivity) of NM. Noninvasive imaging techniques including magnetic resonance imaging (MRI), single photon emission computed tomography (SPECT), positron emission tomography (PET), photoacoustic (PA) imaging, ultrasound (US), X-ray-based imaging modalities (phase-contrast X-ray imaging (PCXI) and computed tomography (CT)), and optical imaging (e.g., in vivo imaging system (IVIS)) each provide different advantages in imaging drug/NM distribution, release, and efficacy.[10,11] MRI provides both morphological and functional information (e.g., target site of NM) in the human lung but is limited by poor spatial resolution (∼1 mm), slow image acquisition, and low sensitivity in the lungs of small animals.[12,13] Nuclear medical imaging including PET and SPECT offers high sensitivity, high imaging depth, and sufficient dosimetric accuracy for quantitative biodistribution and biokinetics studies in the lung. Since those techniques often need a radiolabeled probe and suffer from poor spatial resolution and lack of anatomical information, hybrid imaging techniques have been proposed to overcome the latter limitations (e.g., joint use of SPECT-CT).[10] Taking advantage of the combined spectral selectivity of molecular excitation by laser light and high resolution of ultrasound detection,[14] PA imaging is an emerging nondestructive hybrid technique (optics and acoustics) that allows deeper tissue visualization of fluorescent agents compared to optical imaging. Similar to US, PA can provide anatomical and dynamic imaging, but it is of limited use for the lung due to restricted ultrasound propagation.[15] Consequently, the penetration depth of light, sensitivity, and quantification ability offered by PA imaging are not sufficient for determination of NM biodistribution and pharmacodynamics in the lungs. X-ray-based approaches (PCXI and CT) have been recently applied to monitor the pulmonary fluid delivery dynamics and deposition,[16–18] and lung pathophysiological state[19] with high temporal and spatial resolution. The major challenges posed by X-ray modalities are that they are incapable of assessing NM bioactivity and for visualizing NM at cellular resolution. Leveraging the wide range of bioactive optical probes noninvasive optical imaging could resolve the bioactivity issue, but it suffers from poor spatial resolution and low dosimetric accuracy due to strong tissue scattering and absorption of light. There is no single imaging modality that allows for detection of the dynamics of pulmonary drug delivery, regional distribution profile, accurate dosimetry, and cellular localization of NM in intact organ/tissue of animal models.

Multimodal hybrid imaging techniques such as PET-CT and SPECT-MRI are increasingly used as they provide deeper insight into targeting and distribution profile of NM in terms of detection sensitivity, anatomical information, and spatially resolved dosimetry.[10,11,20] However, most studies have focused on determining the delivery and therapeutic efficacy of NM to xenograft tumor models in murine models,[21,22] while there is still a lack of understanding of pulmonary drug delivery. In spite of a large number of novel-designed NMs with enhanced pharmacokinetic and pharmacodynamic properties,[23] such as non-mucoadhesive particles (particles that do not adhere to mucus) prolonging lung retention for sustained release of drugs in the lung,[24] monitoring the controlled pulmonary delivery of NM in real-time with subsequent analysis of regional spatial distribution, accurate dosimetry, and cellular localization of NM in the lung is still a major unmet need.

This study therefore aims to decipher the dynamics of pulmonary delivery of NPs as well as their regional distribution, dosimetry, and cellular localization by multimodal imaging leveraging X-ray and fluorescence-based imaging techniques. Lung tissue usually produces low contrast in conventional X-ray images, but the visibility of the lungs can be enhanced using phase-contrast techniques that exploit the phase shift introduced by a sample. For weakly absorbing materials such as soft tissue and air, the difference in the phase shift is significant, which leads to contrast enhancement. Propagation-based phase-contrast X-ray imaging (PB-PCXI) was chosen here since this technique requires only a single exposure (compared to, e.g., grating-based phase-contrast imaging), so it is ideal for imaging dynamic processes.[24] Furthermore, it is a simple technique, because the only change with respect to conventional absorption imaging is an increased sample-to-detector distance and increased source coherence. This coherence requirement...
NP delivery with PB-PCXI

2. Results

The multimodal imaging approaches presented here provide unprecedented concomitant insights into dose, distribution, and mechanism of pulmonary delivery of drugs at high temporal and spatial resolution. In vivo PB-PCXI allows for non-invasive time-resolved monitoring of the dynamic process of drug/nanoparticle delivery, while fluorescence-based ex vivo imaging (IVIS and tissue-cleared LSFM) offers quantitative dosimetry and accurate 3D localization of fluorescent tracers (here NPs) with cellular resolution at one time point. Each of these methods requires the presence of either X-ray contrast agents or fluorocative substances. Here a variety of NPs and molecular substances are used, including the clinically approved iodine as well as gold NPs, iron oxide particles, quantum dots (QDs) for X-ray imaging, as well as QDs and polystyrene-SkyBlue and melamine resin NPs for fluorescence-based imaging (Table 1). Except for QDs, which are both X-ray absorbing and fluorescent, all other substances require co-application of two contrast agents. The insoluble particles used here have a diameter between 18 and 900 nm with a relatively narrow size distribution (geometric standard deviation $< 1.4$; Figure S1, Supporting Information).

A schematic overview of the various imaging modalities is presented in Figure 1. During the pulmonary delivery process time-resolved in vivo PB-PCXI was performed and mice were sacrificed immediately after completion of delivery, lungs were perfused (removal of blood), and then excised. Subsequent ex vivo imaging was performed on the very same lungs using either LSFM on tissue-cleared lungs or X-ray imaging (CT scan) followed by two IVIS fluorescence imaging modalities on dried lungs, namely, whole lung fluorescence imaging (WLFI) and lung slice fluorescence imaging (LSFI).

2.1. Time-Resolved In Vivo Monitoring of Controlled Pulmonary NP Delivery with PB-PCXI

For analysis of the mechanisms involved in pulmonary drug delivery (here: iodine-NP) the temporal evolution of drug accumulation in the different regions of the lung was monitored for various forms of drug application in real time by PB-PCXI. The intratracheal instillation and X-ray imaging setup is depicted in Figure 1a. As described by Gradl et al.,[24] mice were fixed in an upright position, mechanically ventilated, and an iodine-polystyrene NP suspension was instilled slowly into the lungs using a remotely controlled syringe pump. This permits delivery of a known amount of liquid at a controllable and constant rate (here 4.2 $\mu$L s$^{-1}$, i.e., 100 $\mu$L within 24 s). Triggered by the ventilator, one image per breath was captured during an end-inspiratory breath-hold phase, which minimizes inflation/deflation-induced blurring effects.[24] This allowed for time-resolved visualization of the pulmonary NP delivery process. Since intratracheal instillation is typically
performed by rapid manual emptying of a syringe (within less than 1 s), we thus refer to the instillation process used here as “slow-instillation.”

Figure 2a shows the X-ray images obtained with this setup. The rib cage and spinal cord of the mouse (dark structures) as well as the lung (bright area within the rib cage) enclosing

Figure 2. Controlled iodine-nanoparticle liquid delivery to the left/right or whole lung via slow-instillation can be visualized in vivo using PB-PCXI. a) A raw X-ray image directly captured during instillation of 100 µL iodine-polystyrene NP liquid to the mouse lung. b) Same as panel (a), but a pre-delivery image is subtracted, clearly enhancing the visibility of the iodine-polystyrene NP liquid in the left half of the lung. c–e) Difference images as well, but for different experiments delivering the iodine-polystyrene NP liquid to the upper right lung, lower right lung, and entire lung, respectively, by controlling the position/depth of intubation cannula. f) Pixel-by-pixel intensity profiles along the linear trajectories across the 2D lung images (along the colored dashed lines in panels (b)–(e)) demonstrate that massive decreases in pixel intensity (compared to baseline in blue) result from the substantial deposition of iodine, while the fluctuations in the signal are due to, e.g., imperfect subtraction of the predelivery image.
the heart (dark region in the top center of the lung region) are clearly visible. By subtraction of the predelivery image from each image during substance delivery a difference image is obtained, which reduces the “background” signal from the rib cage, spine, and heart (Figure 2b). Consequently, this difference image more clearly shows the pulmonary distribution of the iodine-NP liquid, which in this case was directed to the left half of the lung by placing the intubation cannula beyond the first bronchial bifurcation (separating the left and right half of the lung). Moreover, the subtraction image also highlights the position of the main bronchi and boundaries of the lung, which provides guidance on localization of the delivered iodine-NP solution which is useful for data interpretation. Figure 2c–e displays equivalent images for different delivery experiments via intratracheal instillation directed to the right upper, right lower, and whole lung, respectively, via proper positioning of the inserted cannula. Moreover, intensity profiles along (linear) trajectories across the 2D lung images depicted in Figure 2e illustrate the variability of the gray-scale signal for the different regions of these lungs, where lower signals correspond to regions of higher iodine dose (high X-ray absorption), indicating the major locations of iodine-NP liquid (Figure 2f). This pixel-by-pixel analysis (pixel size: 10.4 µm) reveals massive loss of X-ray intensity in regions of substantial iodine deposition, while the fluctuations in signal originate from artifacts due to, e.g., imperfect background subtraction partially due to unavoidable slight position changes of the breathing mouse during the course of the substance delivery process.

Time-resolved in vivo visualization of the dynamic process of liquid delivery via intratracheal instillation to the lung (see Videos S1 and S2 in the Supporting Information for left and whole lung delivery) reveals that the main delivery mechanism is not only gradual spreading of the liquid from the point of entry (trachea/primary bronchus) to the more distal parts of the lung, but also by recurring liquid accumulation in and blocking of one of the airways followed by rupturing of the liquid blockage during breathing activity. This results in secondary spray/aerosol formation, which provides an efficient mechanism for bulk liquid reaching the distal parts of the lung.

2.2. Ex Vivo Multimodal Imaging of NP Distribution in the Lungs

After in vivo PB-PCXI each lung underwent ex vivo imaging using either LSFM or CT scanning followed by WLF1 and LSFI (Figure 1b,c). Both imaging modes are mutually exclusive, since the former requires wet fixation and optical tissue clearing, while the latter involves drying of the lung. Since the last in vivo PB-PCXI image corresponds to the ex vivo images, the agreement between in vivo PB-PCXI and ex vivo imaging modalities can be assessed. Figure 3 presents an example of this data set after slow-instillation of 100 µL iodine-NP solution. The pseudocolored PB-PCXI difference image that is overlaid with a directly obtained image clearly indicates two main regions of iodine-NP deposition, one in the upper right lung and the other around the diaphragm (Figure 3a and Video S3, Supporting Information). 3D reconstructed ex vivo lung from a CT scan of the same subject depicts a qualitatively consistent distribution profile with preferential right superior lobe targeting and some near-diaphragm postcaval lobe deposition (Figure 3b and Video S4, Supporting Information). This distribution pattern is also found by WLF1 (Figure 3c and Figure S2, Supporting Information) and LSFI (Figure 3d).

Ex vivo fluorescence LSFI allows for quantitative analysis of the regional targeting of fluorescence substances. Lungs from nine independent slow-instillation experiments were stratified for preferentially left and right as well as uniform (global) NP deposition in the lung. The ratios of the fluorescence intensities from the right and left lung, which are normalized to their respective right and left lung volume to account for the effect of volume-dependent lung ventilation, reveal that for global delivery the ratio is equal to unity and a 3.7-fold preferential targeting of the right (3.7) and left (3.7 = 1/0.27) half of the lung was accomplished, respectively (Figure 3e). Finally, the delivered dose was determined from quantitative fluorescence analysis using dose-intensity conversion curves based on two reference lungs containing known amounts of polystyrene-SkyBlue NPs (using regular intratracheal instillation) and three nontreated lungs for tissue-autofluorescence correction. In this context it is important to note that the presence of iodine did not affect the fluorescence signal from SkyBlue NPs. The present experiments, 50 and 100 µL were applied via intratracheal slow-instillation at a constant delivery rate of 4.2 µL s⁻¹ and 72.98 ± 36.21% and 79.9 ± 24.1% of the nominal dose was delivered to the lung, respectively (Figure 3f), where for one of the 50 µL lungs almost nothing (<7%) reached the lung probably due to accidental withdrawal of the intubation cannula during the delivery procedure. Hence, no significant difference in deposited fraction of applied dose/volume was found for different volumes via slow-instillation (between 50 and 100 µL). It also reveals that slow-instillation of 50 µL liquid yields the same fractional deposition as reported for regular instillation 77.2 ± 14.2% (50 µL in <1 s; no data for 100 µL are available). Quantitative analysis with any of the other imaging modalities is difficult due to substantial tissue attenuation for whole lung fluorescence imaging and challenges in separating X-ray contrast seen from the treatment and the X-ray contrast seen from the living anatomy.

In summary, this analysis shows that in vivo X-ray and ex vivo X-ray and fluorescence imaging approaches show qualitatively consistent pulmonary deposition patterns for intratracheal slow-instillation, which indicates co-localization of X-ray and fluorescence-active agents. Moreover, LSFI allows for quantitative dosimetry analysis of fluorescent NPs. The data presented here also show that positioning of the intubation cannula beyond the first bifurcation of the bronchial tree allows for regional targeting of liquid substances into the left or right half of the lung, but deliberate selection of either the right or left half is difficult. Finally, regular and slow-instillation provide similar substance delivery efficiency (~75%) with somewhat better dose reproducibility for regular instillation.

2.3. Visualization of Small-Volume Slow-Instillation by Multimodal Imaging

Visualization of extremely small amounts of bulk liquid (4 µL) delivery throughout the entire murine lung with ~1000 µL total
lung volume (600 cm$^2$ epithelial surface area) via intratracheal instillation is notoriously difficult. It has been reported that addition of lung surfactant could facilitate pulmonary delivery both in terms of speed of dispersion and uniformity of distribution as surfactant is known to reduce surface tension of the applied bulk liquid.$^{[30]}$ In this case, the mouse was intubated for syringe-assisted slow-instillation, but breathing spontaneously and in vivo X-ray image acquisition (100 ms per breath) near the end of inhalation was triggered by monitoring the motion of the chest with an optical displacement sensor.$^{[31]}$ In vivo PB-PCXI revealed that delivery of 4 µL of iodine-polystyrene NP liquid resulted in highly localized, small puddles of liquid into either the right or left lung, but not to both sides ($^{[28]}$Figure 4a and Video S5, Supporting Information). Only the proximal bronchial region was reached and addition of surfactant did not affect delivery speed or localization of the liquid as shown in Figure 4d and Video S6 (Supporting Information). The small amounts of iodine delivered to the lungs were below the detection limit of ex vivo CT as seen from Figure 4b,e. On the other hand, ex vivo WLFI was able to detect the fluorescence signal in the left half of the lung (red in Figure 4c,f) confirming the distribution observed with in vivo PB-PCXI. Quantitative dosimetry based on LSFI (Figure 4g) revealed no statistically significant effect of lung surfactant on the delivered dose fraction of 4 µL iodine-NP liquid (with and without surfactant: 0.71 ± 0.19 and 0.53 ± 0.28, respectively). In fact this delivery fraction is also consistent with the corresponding values for 50 and 100 µL of liquid instillation (Figure 3f).

2.4. Real-Time Monitoring of Particle Delivery by PB-PCXI: Gold, Iron Oxide, and QDs Delivery

In the experiments reported above iodine was added to the NP suspension, since the polystyrene NPs cannot be detected by X-ray analysis. Substances with a high atomic number or density, such as iodine, lanthanide, gold, or bismuth-based materials, are preferable to be used as X-ray contrast agents because of their high absorption cross section.$^{[32]}$ Recently, utilization of NP-based X-ray contrast agents has been intensively discussed for diagnostic and molecular imaging, as NPs provide the potential of high loading of contrast agents and the possibilities of surface modification enhancing cell/organ-specific targeting. Traditionally, gold NPs have been used as X-ray contrast agents for, e.g., imaging tumor targeting of NPs via proper surface functionalization after intravenous injection in vivo.$^{[33]}$
However, gold NPs have rarely been used as X-ray contrast agent for pulmonary delivery. This study visualizes the dynamic process of pulmonary delivery of three types of particles (two out of the three are NPs) in real time using in vivo PB-PCXI. Figure S4 (Supporting Information) shows five consecutive PB-PCXI frames revealing the temporal evolution of the NP delivery process during nasal aspiration of 40 µL gold NP suspension (delivered to one nostril of the mouse with a rate of 0.16 µL s⁻¹). The pseudocolored difference images highlight the location of the NP suspension. The gold NP suspension initially forms an increasing drop at the tip of the syringe and—once large and close enough to the nostril—the droplet is suddenly inhaled (aspirated) and deposited in the left half of the nasal airways and eventually draining down passed the glottis into the trachea into the left primary bronchus as observed in Video S7 (Supporting Information) and even into the deep lung. The sequence of images in Figure 5a,b displays the temporal evolution of NP delivery into the deep lung via intratracheal slow-instillation of 100 µL of a mixture of iron oxide and (fluorescent) melamine particles and pure QDs, respectively. Clearly, the iron oxide particles (2.5 mg mL⁻¹) were delivered quite uniformly throughout the targeted left half of the lung (Figure 5a), albeit the image contrast is not as good as for iodine (92.5 mg mL⁻¹), which is at least in part due to the lower mass concentration/dose of iron oxide particles. The QDs were delivered to the whole lung (Figure 5b) and the mechanism of delivery is a relatively slow and continuous transport (several seconds) of a film of particle liquid (position indicated by arrow) from the trachea to the deeper regions of the lung. Also, similar to iron oxide, the lower mass dose of QDs (0.4 × 10⁻⁶ m) results in lower contrast compared with iodine (92.5 mg mL⁻¹). Relatively uniform distribution of QDs throughout the lung into the distal airways and alveoli makes it even difficult to visualize. The dynamic processes of instillation delivery for iron oxide particles and QDs are shown in Videos S8 and S9 (Supporting Information), respectively. Of note, QDs are particularly interesting, as they can serve as contrast agent for both X-ray and fluorescence imaging.

2.5. Cellular Localization of NPs Revealed by Ex Vivo Tissue-Cleared LSFM in Nondissected Lungs

None of the imaging modalities above offers the possibility for 3D visualization of NP localization with cellular resolution throughout the entire mouse lung. While CT scan and LSFI offer 3D imaging capabilities, they do not allow for NP localization within individual cells. We recently demonstrated that ex vivo LSFM allows for quantitative co-mapping of lung architecture and NP deposition with single-cell resolution in nondissected lungs.[27] Here we apply this method to study the pulmonary distribution of 100 µL of an NP suspension after intratracheal slow-instillation. Figure 6 shows the general agreement of the whole lung distribution as observed by PB-PCXI and LSFM for iodine-melamine NPs (Figure 6a,b), iron oxide-melamine NPs (Figure 6d,e), and QDs (Figure 6g,h). At cellular resolution (pixel size <1 µm) melanine NPs were found mainly deposited in the bronchi as well as the bronchioles and proximal part of the alveolar duct (Figure 6c), the so-called proximal acinar region. Similar results were found for iron oxide and (fluorescent) melanine NPs delivery however with a more preferential delivery to the left half of the lung.
distribution (Figure 6d,e). Also the small amount of melamine NPs delivered to the right lung was preferentially located in the distal small airways (bronchioles) and proximal alveolar region (entrance of alveolar sac) (Figure 6f). Finally, Figure 6g displays the QDs distribution profile in the whole lung imaged by PB-PCXI and the regions of high QD dose are indicated by red arrows. It is evident that in this case QDs were distributed to all five lung lobes but did not transport into the peripheral lung reaching only a few localized small alveolar regions (Figure 6i). An animation of the distinct 3D distribution profiles of QDs in whole murine lung measured by LSFM can be seen in Video S10 (Supporting Information).

2.6. Multimodal Imaging for Comprehensive Deciphering of the Characteristics of Aerosol Inhalation

The clinically most relevant route of pulmonary drug delivery is aerosol inhalation. Here an iodine-NP liquid was delivered to the lung via ventilator-assisted inhalation of aerosols. Since individual aerosol droplets are too small for direct imaging (2–4 µm diameter) and their liquid/contrast delivery rate was extremely small (8.6 nL s⁻¹; ~490-fold smaller than slow-instillation), in vivo PB-PCXI is very challenging. In order to visualize the dynamic delivery process of aerosol inhalation, a large volume of NP-iodine liquid was nebulized (225–300 µL) and quantitative fluorescence spectroscopy in tissue homogenates revealed that 3.7 ± 1.1% and 5.6 ± 2.3% of the applied volume was deposited in the lung lobes and the trachea, respectively (Figure 7). It is noteworthy that from quantitative spectrofluorometry of the lung slices (LSFI) one obtains a deposited aerosol fraction of 15.6 ± 2.8 % (whole lung) (Figure 7j), which is significantly larger than the 9.3 ± 2.3% (trachea + lung lobes) measured in lung/trachea homogenates, probably due to the higher light attenuation in sliced lung tissue. Since we consider tissue homogenization as more accurate method of LSFI,[27,34] we report 8.6 and 12.9 nL s⁻¹ as pulmonary and tracheal aerosol deposition (delivery) rate, resulting in 5.6 and 8.3 µL NP liquid out of applied 150 µL volume deposited in the lung and trachea, respectively. Not surprisingly, in vivo PB-PCXI showed no iodine-related signal during aerosol application (Figure 7a) due to the low iodine delivery rate and the extremely uniform distribution of (aerosolized) iodine throughout the lung. On the other hand, gradual accumulation of iodine in the lung over the entire inhalation period (10 min and 48 s) was sufficient for obtaining a clear iodine signal from in vivo PB-PCXI (Figure 7a–d and Video S11, Supporting Information).

Moreover, in vivo PB-PCXI was useful to resolve unexpected, nonaerosol related secondary liquid re-distribution mechanisms, which can occur during ventilator-assisted aerosol inhalation. Unlike instillation, which shows a relatively patchy liquid distribution in the lung (Figure 6b), aerosol inhalation spreads the relatively small lung-deposited dose uniformly throughout the entire lung as can be clearly seen from ex vivo 3D reconstruction of the lung from CT scan (Figure 7e), ex vivo WLFI (Figure 7f), and ex vivo tissue-cleared LSFM (Figure 7g–i). During the process of ventilator-assisted inhalation localized accumulations of liquid were observed in the upper airways.
near the trachea (Figure 7b, arrow), which were gradually transported to the lower airways (Figure 7c, arrow) and into the bronchiolar region where they dispersed (Figure 7d, arrows) into even smaller liquid accumulations as evidenced by fluorescence “hot spots” seen in LSFM (Figure 7h, arrows). Not only the presence but also the origin of these occasional accumulations of liquid in the lower bronchial region can be understood from multimodal imaging. Ex vivo whole lung imaging (Figure 7f,g) and quantitative fluorometric dosimetry (Figure 7j) provide evidence for substantial aerosol deposition in the trachea (%60% of the total deposited aerosol dose is deposited in the trachea). Since the murine trachea is a rather narrow duct, it is conceivable that the trachea-deposited aerosol will eventually block the trachea resulting in spillover of some of the liquid in the trachea into the upper airways and from there into the bronchiolar region as observed by in vivo PB-PCXI (Figure 7b–d) and ex vivo LSFM (Video 12, Supporting Information). As this spillover typical occurred after 95 s during nebulization, we conclude that at least 1.2 µL (12.9 nL s−1 × 95 s) of liquid has to accumulate in the trachea for occurrence of the spillover, which corresponds to 22 µL of nebulized liquid (= 1.2 µL/0.056).

3. Discussion

Aerosol delivery of drug/NM is the most widely used application route for treatment of lung disease.[35,36] Targeted delivery of drug/NM on a regional and even cellular scale is of central importance for precision medicine especially for localized lung diseases such as lung cancer, asthma, emphysema, or COPD.[3,37] Also inhalation of ambient NPs is one of the main health hazards associated with NPs.[38,39] The region of NM/NP...
deposition can have a significant impact on residence time, release kinetics, and hence bioactivity of the drug or toxicity of NPs.\[40,41\] While NM/NP can be quickly (within minutes) removed from the upper airways by mucociliary transport, clearance of NM/NP from the alveolar epithelium mainly relies on uptake by alveolar macrophages (takes a few hours), which may be enough time for direct bioactivity/toxicity of the NM/NP or result in secondary drug release into or from the macrophages.\[2\] Consequently, understanding the mechanisms of pulmonary NM/NP delivery and their relevance for tissue-delivered NM/NP dose with cellular resolution is essential for the development of effective NM\[42\] or minimizing health hazards of NPs.\[43\] Standard histological analysis or single-modality imaging techniques cannot resolve the entire delivery process in all relevant temporal (seconds to days) and spatial scales (whole lung to cellular).\[44\] This is also the case for the preclinical efficacy or toxicity testing of NM/NP using animal models.

In this study we combined in vivo X-ray and ex vivo fluorescence imaging modalities to investigate the dynamic process of pulmonary NM delivery and its impact on pulmonary NM distribution with high temporal and spatial resolution in murine lungs. PB-PCXI utilizes differences in X-ray refractive index and associated phase shifts at air–tissue interfaces for enhancing soft tissue contrast (e.g., in the lung) as compared to X-ray absorption.\[26\] Building on our previously published in vivo PB-PCXI studies in murine lungs,\[18,24\] the present study demonstrates that combined in vivo PB-PCXI and ex vivo fluorescence imaging can provide complementary information, which enhances the understanding of complex processes such as NM/NP delivery to the lung in a quantitative way on various scales (whole lung to cellular). The strengths and weaknesses of all five imaging modalities used here are summarized with respect to 2D/3D imaging capability, resolution, fidelity (conservation of original anatomical 3D structure), anatomical information, and technical complexity as summarized in Table 1. In general, X-ray imaging is used here for in vivo imaging at high temporal resolution (1 Hz), while ex vivo fluorescence imaging provides insight into spatially resolved dosimetry.

In preclinical lung research, instillation or aspiration of bulk liquids is the most widely used method of pulmonary drug delivery. On the other hand, aerosol inhalation—not bulk liquid application—is the preferred route of drug delivery in clinical settings. In spite of their widespread use detailed information on the dynamic details of the application processes and spatially resolved dosimetry is scare. All three application routes have been investigated in this study, but a significant focus was on ventilator-assisted aerosol inhalation, since it offers a technologically mature, efficient (≈4% of invested dose is delivered to lung) and dose-controlled method for physiologic delivery of liquid substances to the lungs of animal models (here mice) using a commercially available device (flexiVent system, Small 2019, 1904112).
EMKA/SciReq Inc.). As such it has the potential to at least partially replace intratracheal instillation and nasal aspiration in future preclinical inhalation drug or toxicity testing.

It is evident that intratracheal instillation allows for highly efficient (76.3 ± 23.0% of invested dose reaches the lung), dose-controlled delivery of liquids (repeatability: 30.1%) for all tested liquid volumes (4–100 µL) into the deep parts of the lung (bronchioles and even alveoli). It is not intuitively clear how a bulk liquid injected into the trachea via intratracheal instillation can reach the deeper parts of the lung. In vivo PB-PCXI revealed that the instilled bulk liquid is not primarily flowing down the bronchial tree, but is distributed quite uniformly throughout the lung by secondary aerosol formation due to occasional blockage of the airways with liquid and subsequent bursting of this blockage during breathing activity (Videos S1–S3, Supporting Information).[24] As mentioned above, the slow-inflation process (24 s for 100 µL; 4.2 µL s⁻¹), which was used here for the sake of in vivo PB-PCXI imaging, is not identical to the typically used, rapid manual instillation (<1 s, >50 µL s⁻¹).

However, the main features of pulmonary fluorescence distribution and the measured delivery efficiency (76.3 ± 23.0%) are in excellent agreement with observations for rapid instillation (77.2 ± 14.2%) as provided by Barapatre et al.[29]

In vivo PB-PCXI also provides valuable insights into the mechanisms of pulmonary NM/NP delivery via ventilator-assisted aerosol inhalation. As expected there is a very gradual, uniformly distributed increase in X-ray signal with no burst-like events as seen for intratracheal instillation, which is related to aerosol deposition in the lung occurring uniformly at a relatively low delivery rate. However, even for ventilator-assisted aerosol inhalation we observed occasional localized accumulations of liquid in the upper airways near the trachea, which were gradually flowing down the airway tree reaching even the bronchiolar region (Figure 7b–d). Fluorescence imaging and fluorescence-based dosimetry revealed that a large fraction of the aerosol dose is deposited in the trachea (~60% of total deposited dose) due to the spray-like aerosol stream exiting the narrow intratracheal intubation cannula. Since direct aerosol deposition in the bronchial tree would result in a more gradual process of liquid accumulation, spillover from the trachea is the most likely reason for this to occur. From the measured deposition rate (based on fluorescence) and the time of first occurrence of liquid accumulation in the airways (in vivo PB-PCXI), we determined that this nonaerosol-related transport effect occurs if more than 22 µL of liquid is nebulized. The critical liquid volume of 22 µL is expected to depend on positioning of the mouse and on ventilation parameters (breathing frequency, tidal volume, length of breath hold, and inhalation-to-exhalation time ratio). In our case, the mouse was held in upright position (for the sake of in vivo PB-PCXI), while for typical aerosol applications the mouse is laying horizontally on its back. Therefore, we recommend nebulizing as little volume as possible with the flexiVent ventilation system to avoid nonaerosol-related lung delivery processes. However, even with this artifact the delivery of NPs to the lung is much more uniform with ventilator-assisted aerosol inhalation than with intratracheal instillation on a whole lung, regional, generation, and cellular scale. It is important to note that the initially unexpected fluorescence “hot spots” detected with LSFM in the deeper bronchial region (Figure 7h) could not have been explained without the information provided by in vivo PB-PCXI.

A prerequisite for combing X-ray and fluorescence imaging is that the co-applied active agents (X-ray absorption and fluorescence activity) have the same deposition profile in the lung. Since both agents are applied as liquid mixture, this is to be expected. This was confirmed for all contrast/fluorescence agents/mixtures used here (Table 1)—not just the clinically approved iodine—as evidenced by the excellent qualitative agreement of the spatial distribution profiles for all imaging models covering different volumes (4–100 µL), different localization (left, right, whole lung), and different degrees of spatial uniformity (patchy or uniform) of the applied liquid. QD is the only type of contrast agent (NP) that is both X-ray and fluorescence active.

Limitations of the X-ray imaging modalities were mainly related to the signal-to-noise ratio for low amounts of liquid or very uniformly distributed contrast agents. For instance ex vivo CT reached its detection limit for localized delivery of 4 µL (iodine-NP solution with 92.5 mg mL⁻¹ iodine) of bulk liquid and uniformly distributed aerosol (11 µL) (Figure 4b,d and Figure 7e). Also in vivo PB-PCXI was difficult, but not impossible, for the latter case. On the other hand, fluorescence imaging on dried lungs (especially on whole lungs; WLFI) was prone to widen the apparent region of particle deposition due to tissue-related light scattering effects, which can be largely eliminated by tissue clearing combined with LSFM.

As mentioned above, most of the standard imaging modalities (e.g., PA, US, CT, SPECT, PET, and MRI) are unable to visualize pulmonary fluid delivery dynamics at 1 Hz under in vivo conditions in mice mainly due to the poor spatial and temporal resolution.[16] Recently, some multimodal imaging techniques have been used for determination of NP biodistribution and bioactivity in preclinical research. For instance, a combination of triple imaging techniques (i.e., dark-field light microscopy, electron microscopy, and nanoscale secondary ion mass spectrometry) was used to study the NP interaction with green algae in the context of NP localization, internalization, and chemical identity.[45] Also, quantitative imaging of co-localization of tumor associated macrophages with therapeutic ⁶⁷Cu-labeled polyglucose nanoparticle in an orthotopic model of lung adenocarcinoma was accomplished via PET, in vivo confocal microscopy, and tissue-cleared LSFM.[46] PCXI has already been applied for ex vivo and in vivo studies on mucociliary transport of large microparticles or 5–100 µm fibers in the trachea or upper airways of animal models such as mice and pigs[26,47–49] and for monitoring the delivery of liquids to murine lungs via nose or intubated cannula delivery.[16–18,25] Moreover, several common bulk particulates (e.g., lead dust, quarry dust, glass beads, asbestost, and Galena with size ≥ 5 µm) were tracked in live animal trachea airways by PCXI, showing the high variability in particle movement during mucociliary transport.[26,47,50] A few available NPs with high atomic number such as gold NPs, bismuth NPs, lanthanide-doped NPs, and tantalum pentoxide NPs as new contrast agents of X-ray-based modalities have also been studied for diagnostic and molecular imaging.[13] However, to the best of our knowledge, real-time monitoring of the pulmonary delivery of NP suspensions has not been studied yet, and the combination of in vivo PCXI and quantitative and spatially
resolved ex vivo fluorescence imaging provided complementary insights into the mechanisms, regional/cellular distribution, and dosimetry of pulmonary drug delivery in mice. By virtue of bioactive fluorescent probes fluorescence imaging is also suitable for bioactivity studies. In principle the five modes of imaging presented here can be reduced to two modalities, namely, in vivo PB-PCXI and one of the three fluorescence imaging modalities, which should be selected based on the experimental constraints (available time for sample and image processing, cost, spatial resolution, etc.).

The core issue of this study is to decipher the main features of the dynamic delivery process of NPs to the nose/lung and to characterize the initial 3D NP distribution profile immediately after varied routes of pulmonary delivery. Here, NPs were suspended in liquid and delivered either as bulk liquid (nasal aspiration, intratracheal instillation) or droplets (aerosol inhalation). Consequently, the initial deposition profile of NPs mainly depends on liquid properties (e.g., delivery rate and droplet size). The more patchy, central deposition of QDs (Figure 6g) as compared to melamine NPs (Figure 6b) could be related to the higher viscosity of QD suspensions. After deposition on the lung epithelium the liquid phase of the NP suspensions will be resorbed by lung tissue and re-distribution of NPs within the lung tissue-cleared lungs. While each of these imaging modes has strengths and weaknesses, selection of PB-PCXI and LSFM would not result in substantial loss of information. Mixing of particles as contrast agents for X-ray (iodine, gold NPs, iron oxide particles, and QDs) and fluorescence imaging (fluorescently labeled polystyrene or melamine NP and QDs) allowed for time-resolved (1.5 Hz) in vivo visualization of the dynamics of the pulmonary particle delivery process via intratracheal slow-instillation or ventilator-assisted aerosol inhalation with in vivo PB-PCXI. Moreover, in vivo and ex vivo X-ray particle distribution profiles were in good qualitative and quantitative agreement with regional distribution patterns observed with fluorescence imaging techniques. This implies that the two applied contrast are co-localized during and directly after delivery. Our study also reveals that for intratracheal instillation of a bulk liquid the pulmonary distribution does not primarily occur via draining of the liquid down the bronchial tree, but by secondary aerosol formation associated with liquid blockage of airways and subsequent bursting of this blockage during breathing activity. In contrast, time-resolved in vivo imaging of the ventilator-assisted aerosol inhalation process revealed slowly accumulating uniform aerosol deposition throughout the lung with occasional transport of bulk liquid into the lung.

The latter is likely due to direct impaction of aerosol spray exiting the intubation cannula resulting in liquid accumulation in the trachea and eventual spillover into the bronchial region of the lung, if more than 22 µL of liquid is nebulized. This spillover explains the initially unexpected sporadic occurrence of 100–200 µm hot-spot regions in ex vivo LSFM lung images after ventilator-assisted inhalation. Development of bimodal contrast agents like QDs, which are suitable for both in vivo PXCI and ex vivo LSFM, is expected to be particularly useful for co-visualization of controlled pulmonary delivery of NP in real time and 3D biokinetics over long term. The present study thus describes a complementary multimodal imaging platform for high temporal and spatial resolution visualization of pulmonary NP delivery in a quantitative manner, which will accelerate the ability to target active NM/drugs to the diseased region of the lung and enhance the development and efficacy of the novel-designed NM.

4. Conclusion

In this proof-of-concept study, we introduced and validated a multimodal imaging approach for comprehensive understanding of the mechanisms of pulmonary NP delivery in the context of dynamics of the delivery process, deposited dose, regional distribution, and cellular localization of NP in the (nondissected) whole murine lung. This imaging platform consists of in vivo PB-PCXI, ex vivo CT, and three ex vivo whole and sliced-lung epifluorescence imaging or ex vivo LSFM on tissue-cleared lungs. While each of these imaging modes has strengths and weaknesses, selection of PB-PCXI and LSFM would not result in substantial loss of information. Mixing of particles as contrast agents for X-ray (iodine, gold NPs, iron oxide particles, and QDs) and fluorescence imaging (fluorescently labeled polystyrene or melamine NP and QDs) allowed for time-resolved (1.5 Hz) in vivo visualization of the dynamics of the pulmonary particle delivery process via intratracheal slow-instillation or ventilator-assisted aerosol inhalation with in vivo PB-PCXI. Moreover, in vivo and ex vivo X-ray particle distribution profiles were in good qualitative and quantitative agreement with regional distribution patterns observed with fluorescence imaging techniques. This implies that the two applied contrast are co-localized during and directly after delivery. Our study also reveals that for intratracheal instillation of a bulk liquid the pulmonary distribution does not primarily occur via draining of the liquid down the bronchial tree, but by secondary aerosol formation associated with liquid blockage of airways and subsequent bursting of this blockage during breathing activity. In contrast, time-resolved in vivo imaging of the ventilator-assisted aerosol inhalation process revealed slowly accumulating uniform aerosol deposition throughout the lung with occasional transport of bulk liquid into the lung. The latter is likely due to direct impaction of aerosol spray exiting the intubation cannula resulting in liquid accumulation in the trachea and eventual spillover into the bronchial region of the lung, if more than 22 µL of liquid is nebulized. This spillover explains the initially unexpected sporadic occurrence of 100–200 µm hot-spot regions in ex vivo LSFM lung images after ventilator-assisted inhalation. Development of bimodal contrast agents like QDs, which are suitable for both in vivo PXCI and ex vivo LSFM, is expected to be particularly useful for co-visualization of controlled pulmonary delivery of NP in real time and 3D biokinetics over long term. The present study thus describes a complementary multimodal imaging platform for high temporal and spatial resolution visualization of pulmonary NP delivery in a quantitative manner, which will accelerate the ability to target active NM/drugs to the diseased region of the lung and enhance the development and efficacy of the novel-designed NM.

5. Experimental Section

By virtue of concomitant multimodal in vivo and ex vivo imaging new insights into key aspects of common preclinical and clinical methods for pulmonary substance delivery are presented. The related materials and methods are described including the diverse set of imaging modalities and the panel of methods for pulmonary substance delivery.

Materials: Each of the imaging modalities required the use of materials with specific X-ray contrast or fluorooactivity.

For X-ray phase-contrast imaging, a clinically approved iodine-based contrast agent (Ultravist-370, 370 mg iodine per milliliter) and three types of metal NPs were used, namely, gold NPs ((volume median diameter (VMD)) 20.7 ± 9.7 nm), Roti-MagBeads (magnetic iron oxide particles, and QDs) and fluorescence imaging (fluorescently labeled polystyrene or melamine NP and QDs) and fluorescence imaging (fluorescently labeled polystyrene or melamine NP and QDs). In this study, 20 nm QDs were successfully applied as an example for a bimodal NP. Alternatively, encapsulated or conjugated hybrid particles consisting of one fluorescent and one X-ray-absorbing material, such as iodinated polymers or liposomes with fluorescent dyes that have been widely used for bimodal imaging of NP accumulation in tumors, could be particularly useful in future pulmonary studies.
of a 0.04 mol L\(^{-1}\) aqueous solution of sodium citrate (HOC(COONa)(CH\(_3\)COONa)\(_2\); 2H\(_2\)O, ≥99.0%, Sigma-Aldrich) was added to the gold solution, which was kept boiling for further 5 min. The solution changed color from transparent yellow to transparent gray, then black, and finally dark pink. Subsequently, the solution was taken from the heating plate and left stirring for 1 h in the dark, and then stored in the dark until use. The purchased QDs (stock solution: 8 \(\times\) 10\(^{-6}\) w) were made from the crystals of a semiconductor material (CdSeTe), shelled with a ZnS layer, and further coated with a polymer layer with carboxylic groups.

For fluorescence-based microscopy, polystyrene NPs with the embedded fluorescent dye SkyBlue (excitation/emission (ex/em); \(\approx 470\) nm/\(\approx 515\) nm; VMD: 345 nm; 90\% transmittance) were used. To measure dynamic light scattering using a Malvern Zetasizer Nano instrument (Malvern Instruments Ltd., Malvern, UK) (Figure S1, Supporting Information). After mixing (Figure S3, Supporting Information), the fluorescence signals, which remained stable for more than a week (from about 490 to 770 nm) (Figure S1, Supporting Information) were detected. Polystyrene NPs were dispersed in water and further coated with a polymer layer with carboxylic groups. When transferred from water to iodine solution, the observed increase of about 50\% in VMD of polystyrene NPs was timed.

### Sizing of Nanoparticles

#### Suspension: Hydrodynamic diameter measurement of all NP suspensions was performed using dynamic light scattering using a Malvern Zetasizer Nano instrument (Malvern Instruments Ltd., Malvern, UK) (Figure S1, Supporting Information). Of note, the observed increase of about 50\% in VMD of polystyrene and melamine NPs when transferring them from water to iodine solution (from about 490 to 770 nm) (Figure S1, Supporting Information) was noted due to the absence of a breath-hold phase. It is noteworthy that for practical reasons the liquid delivery rates, 4.0 and 0.8 µL s\(^{-1}\) are slow compared to typical intratracheal instillation protocols delivering 50–200 µL within <1 s; by manually emptying a loaded syringe via the intubation cannula into the trachea at a delivery rate of >50 µL s\(^{-1}\). Since less than 1 s of delivery is too short for reliable in vivo PB-PCXI imaging of different stages of the delivery process, slower delivery rates were chosen here.

#### Animal Handling: Wildtype C57BL/6 female mice (age 9–18 weeks, 19–25 g) were instilled in individually ventilated cages (IVC-Racks; Bio-Zone, Margate, UK) supplied with filtered air in a 12 h light/12 h dark cycle. Mice were provided with food (standard chow) and water ad libitum. All procedures involving animal handling and experiments were carried out in accordance with protocols approved by the Regierung von Oberbayern (District Government of Upper Bavaria, AZ55.2-1-54-2532-108.13).

The animals were anesthetized by intraperitoneal injection of a tripe combination of medetomidine (0.5 mg kg\(^{-1}\) body weight), midazolam (5 mg kg\(^{-1}\) body weight), and fentanyl (0.05 mg kg\(^{-1}\) body weight). The mice were then intubated by a nonsurgical technique using a 20 G cannula with one side inserted into the trachea and the other side connected to a mechanical ventilator (flexiVent FX system, Scireq Inc., Canada), allowing for exact control over the breathing activity of the mouse during pulmonary application of liquids and during in vivo acquisition of images.

#### Methods of Pulmonary Substance Delivery

Three widely used techniques of pulmonary delivery of liquid substances were investigated here, namely, intratracheal instillation and nasal aspiration of bulk liquids, as well as (ventilator-assisted) inhalation of aerosolized liquids. For these methods time-resolved in vivo monitoring of pulmonary substance delivery throughout the whole lung was performed with PB-PCXI, which is described in detail below. However, the technical details related to PB-PCXI image acquisition deserve careful attention and are therefore included in this section.

For “intratracheal slow-instillation” delivery, the setting was similar to that depicted in previous studies.\(^{[24,25]}\) The mice were mechanically ventilated at 90 breaths per minute, 30 mL kg\(^{-1}\) tidal volume, and an inhalation–expiration (breath-hold)–expiration time ratio of 1:1:1. The liquid was introduced by a heat thinned PE10 polyethylene tube that was passed through the flexible wall of the ventilator inspiratory tube. The inner PE tube was small enough not to block the intubation cannula enabling simultaneous liquid delivery and mechanical ventilation. The liquid delivery (slow-instillation) was remotely controlled by a syringe pump (UltraMicroPump III and Micro4 controller, World Precision Instruments, Sarasota, FL) that allowed for both controlled liquid delivery from outside the imaging hutch and consecutive imaging during the whole instillation process. As listed in Table 1, 100 µL of various NP mixtures for combined X-ray and fluorescence imaging (SkyBlue-iodine: 1:20 SkyBlue (stock suspension) and 1:4 iodine; melamine-iron oxide: 1:20 melamine and 1:4 iron oxide NPs) or QDs (1:20 dilution) was continuously delivered via the syringe pump to the trachea in ~24 s (4.2 µL s\(^{-1}\)). PB-PCXI image acquisition was performed triggered by the ventilator at a frame rate of up to 1.5 fps (frames per second) using a 200 ms (100 ms for QDs) exposure during an end of inspiration 222 ms breath-hold phase.

To study the effect of alveolar surfactant (Alveofact 45 mg mL\(^{-1}\), Lyomark Pharma GmbH, Germany) on the pulmonary distribution of small amounts of liquids applied via intratracheal instillation, the delivery of a small volume (4 µL in 3 s, i.e., 0.8 µL s\(^{-1}\)) of an iodine-NP-surfactant mixture was investigated. The mixture contained 1:4 diluted iodine and 1:20 diluted polystyrene-SkyBlue NPs with (+) or without (−) surfactant (25 mg mL\(^{-1}\)) in distilled water. For this experiment the mice were spontaneously breathing so the ventilator was replaced with a noncontact fiber optic displacement sensor (RC-60, Phitec, MD) that was configured to detect respiratory motion and deliver an electrical trigger signal for image capture. The frame rate was maintained at 1.5 fps, but the exposure time was reduced to 100 ms to mitigate unavoidable motion blur due to the absence of a breath-hold phase. It is noteworthy that for practical reasons the liquid delivery rates, 4.0 and 0.8 µL s\(^{-1}\) are slow compared to typical intratracheal instillation protocols delivering 50–200 µL within <1 s; by manually emptying a loaded syringe via the intubation cannula into the trachea at a delivery rate of >50 µL s\(^{-1}\). Since less than 1 s of delivery time is too short for reliable in vivo PB-PCXI imaging of different stages of the delivery process, slower delivery rates were chosen here, and image acquisition was performed as described above.

For “ventilator-assisted aerosol inhalation,” a similar protocol and setup as described for 100 µL instillation was used (80 breath per minute, 30 mL kg\(^{-1}\) tidal volume, 200 ms) and PB-PCXI image acquisition occurred during an end-inspiratory breath-hold phase as described above. In contrast to intratracheal slow-instillation, the inner PE10 polyethylene tube was removed and the ventilator was equipped with a nebulizer (Aeroneb Lab Small, Aerogen Inc., Galway, Ireland) in the inspiratory tubing for the generation and transport of liquid aerosol droplets to the lungs of mice. It was shown that for the flexiVent system operated under the conditions described above about 4%-5% of the nebulized liquid was deposited in the lung (downstream of trachea), with the rest being deposited in the ventilation tubing or exhaled by the mouse.\(^{[27]}\) Moreover, it was determined empirically that 150 µL was the maximum liquid volume that could be nebulized without at least partially blocking some part of the inspiratory ventilation tube by accumulated rainout. Here, the nebulizer typically generated droplets with a liquid output rate of 0.26 mL min\(^{-1}\) and a volume-weighted median diameter of 2–4.0 µm. Here, the nebulizer was breath activated and active for 40 ms during the inspiratory phase. For a breathing rate of 80 breaths per minute this corresponded to an effective liquid output rate of 13.8 µL min\(^{-1}\) during flexiVent operation. Hence it took ~10.8 min (870 breaths) to nebulize 150 µL liquid resulting in 5.6 µL of contrast agent deposited in the lung (3.7% of applied dose was deposited in the lung), where the dosimetric results from quantitative fluorescence spectroscopy in lung homogenates rather than the experimentally more uncertain LSFI results were relied on.\(^{[19]}\) (Figure 7g). The aerosol delivery rate to the lung was 8.6 mL s\(^{-1}\) (≈0.0086 mL s\(^{-1}\)) = 5.6 µL/10.8 min). For some experiments, a second nebulization of 75 or 150 µL of liquid was carried out keeping the animal ventilated while drying the ventilation tube between nebulizations. For these experiments a 1:16 polystyrene NPs and 1:5 iodine mixture in distilled water was used.

For “nasal aspiration,” a similar protocol as applied was reported by Gradl et al.\(^{[18]}\) Briefly, a mixture of polystyrene NPs (1:10 dilution) and gold NP (1:2 dilution) suspensions was loaded into a syringe pump mentioned above, permitting controlled liquid delivery outside the imaging hutch. Here 80 µL of the polystyrene-gold liquid mixture was spontaneously inhaled via the nose by the mouse. The PB-PCXI frame rate was maintained at 1.5 fps with the exposure time of 100 ms.
In Vivo PB-PCXI: All X-ray imaging experiments were carried out at an inverse Compton scattering source—the MuCLS located at the Technical University of Munich (TUM) in Garching, Germany. The source itself was developed and installed by Lyncan Technologies Inc. (Fremont, USA)\textsuperscript{[24,54]} and the X-ray beamline infrastructure was designed and developed by TUM researchers. The source delivered a quasi-monochromatic and low divergence (4 mrad) X-ray beam. 25 keV X-rays with flux up to $2 \times 10^{10}$ ph s$^{-1}$ were used. The principle behind propagation-based phase-contrast imaging was to use free space propagation of a partial spatial coherent beam (typically around 1 m) after penetrating a sample to introduce additional contrast. As the x-ray wavefield propagated from the sample to the detector, the amplitude- and phase-modulated waves combined, resulting in an interference pattern being formed at the detector. This interference pattern was then converted into a quantitative measurement of the deposited dose in those lungs could be achieved. The left/right/whole lung deposition analysis was carried out in the ex vivo WLI data. The background intensity was subtracted to yield the absolute fluorescence intensity of SkyBlue for each part and the fluorophore intensity could be compared.

Ex Vivo Tissue-Clearing LSF: Other non-dried lung samples were optically cleared with a modified version of the 3DISCO protocol\textsuperscript{[27]} as described in our recent study.\textsuperscript{[27]} Briefly, lung samples were dehydrated overnight in 10 mL of 50% v/v tetrahydrofuran/H$_2$O (THF, Sigma 186562-1L), 50% THF/H$_2$O 1 h, 70% THF/H$_2$O 4 h, 80% THF/H$_2$O 4 h, 100% THF 1 h, 100% THF overnight, and 100% THF 1 h with slightly shaking. Samples were gently dried and then incubated in dichloromethane (Sigma 270997-1L) around 30 min until they sank to the bottom. Finally, samples were incubated without shaking in dibenzyl ether (DBE, Sigma 108014-1KG) for at least 2 h until imaging and then stored in DBE at room temperature.

Lung samples were scanned with an LSFM (Ultramicroscope II, LaVision) equipped with a sCMOS camera (Andor Neo) and a 2x objective lens (Olympus MVP/LAPO 2x/0.5 numerical aperture (NA) equipped with an Olympus MXV-10 zoom body, which provided zoom-out and -in ranging from 0.63x up to 6.3x. For the whole lung imaging, light sheet scans were generated with 0.63x zoom magnification with individual ex/em bandpass filters for melamine NPs (ex/em = 640(30) nm/690(50) nm) and QDs (ex/em = 640(30) nm/795(50) nm) with a step size of 10 μm. Tissue autofluorescence was imaged under the ex/em = 545(30) nm/605(70) nm filters. Samples were typically imaged with an exposure time of 150 ms, at a full laser power with the light sheet by adjusting xy width and NA to the sample size. High magnification scans (e.g., 8x in Figure 6e,f) were usually performed with a step size of 4 μm. The imaging chamber was filled with DBE.

Image Processing and Analysis: All (X-ray and CT) images were flat field and dark current corrected. X-ray images were analyzed and the dynamic NP delivery videos were generated using ImageJ (https://imagej.nih.gov/ij/). Fluorescence images scanned by IVIS consisting of whole lung images and dried lung slices and their corresponding NP dosimetry analysis were performed with the Living Imaging 4.0 Software (Caliper). The 3D images with maximum intensity projection and videos with 3D manipulation scanned by LSFM were processed using Bitplane Imaris. All data were presented as mean ± standard deviation and * $< P< 0.05$, ** $< P< 0.01$, and *** $< P< 0.001$, and plotted using SigmaPlot version 12.0 (Systat Software GmbH, Germany). N $\geq$ 3.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Keywords
aerosol inhalation therapy, lung fluorescence imaging, optical tissue clearing, pulmonary delivery, X-ray imaging

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