nature portfolio

Peer Review File

Phase-contrast X-ray tomography resolves the terminal bronchioles in free-breathing mice

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The manuscript presents three-dimensional in-vivo x-ray imaging of the lung from a free-breathing mouse using laboratory equipment (not a synchrotron radiation source), which is a great achievement. The manuscript is well written, clearly structured and the authors' conclusions are supported by the presented results. However, several important aspects are either not clearly stated or missing in the current manuscript and should be addressed in a minor revision before publication.
1. The authors mention that the resolution is limited by the detector and the source size. It is not entirely clear to me, whether the blur from the source and the detector were matched. If the detector limits the resolution, one could probably slightly reduce the magnification accordingly, or in case the source is limiting the resolution, one could measure with a binning factor to achieve better statistics. This should be clarified in the manuscript.
2. A Xineos-2329 mammography CMOS flat panel detector (Teledyne DALSA, CA) was used for the measurements. What sensor material does this detector use and how thick is the sensor? This is interesting to be able to judge on the dose efficiency of the presented results. Also, it would be very interesting to see a histogram of a raw projection to see how many counts the detector pixels collect during the 100 ms exposure. Are the images taken at the lowest limit of the dynamic range of this detector? What is the x-ray flux at these settings? What improvement in dose efficiency can be expected when using a photon-counting detector? Would the resolution still be high enough to resolve the edge enhancement for the phase-contrast imaging?
3. The detector has a huge number of pixels (4608 x 5890 pixels). It is not absolutely clear from the manuscript, whether the projections were binned or not? By taking only 720 projections of the full detector image, the undersampling would be enormous. How many pixels on the detector are covered by the magnified sample?
4. Were the projections of the resolution phantom shown in figure 3a also phase retrieved using the Paganin algorithm? Without the phase retrieval the comparison with the reconstructed slide would not be fair, as the edge enhancement would most likely improve the resolution in the projections. This should be clarified in the text.

Reviewer #2 (Remarks to the Author):

In the manuscript "Phase-contrast X-ray tomography resolves the terminal bronchioles in free-breathing mice" the authors describe some proof-of-principle 3D imaging results of the tracheobronchial tree in free-breathing mice without mechanical ventilation. The results are very interesting for a broader biomedical community as they have been obtained at radiation levels compatible with longitudinal studies (i.e. > 500 mGy), and also within a small scale laboratory (in contrast to previous research at large-scale SR facilities).

From a technological and physics point of view the manuscript is original and presents a very important step forward to allow non-invasive longitudinal studies of native state murine airways in a laboratory close to biomedical research facilities. Particularly in view of the rising importance and threat of respiratory diseases worldwide (including COVID-19) this work is very attractive.

The manuscript is generally very well written and the technical details, figures, and description of

analysis is excellent. I appreciate particularly the solid analysis of resolution and discussing of the result in view of existing literature.

While my overall impression is therefore very positive (I highly recommend publication of this manuscript), I have a few smaller (optional) points that the authors might want to consider:

1) Discussion of clinical impact: I fully agree that resolving the tracheobronchial tree down to the terminal bronchioles will enable very important biomedical research studies to better understand diseases progression/treatment/etc. in connection with some lung diseases. It might, however be better suited for those diseases that have more impact on the bronchioles, rather than diseases that mainly/first affect the alveoli (which cannot be resolved at the moment). Good examples here might be small-airway disease, lung fibrosis, or actually COVID. Not so good examples are COPD and actually mechanical ventilation induced injuries (which mainly start in the alveoli). Please add a few sentences to the MS that discuss these potential clinical applications and for what it might best be suited at first.
2) Please discuss in more detail that the heart beat still degrades resolution and that this is worse the closer you get to the heart. Fig. 4a shows exactly that, and if the authors would have chosen a zoom in closer to the heart, this would also look a little more blurred. Just be clear on what is possible and what not (in 3D).
3) In the introductory part, probably the best manuscripts to link to darkfield imaging might be Schleede et al, PNAS, 2012 (https://www.pnas.org/content/109/44/17880) and Gradl et al. IEEE TMI (https://ieeexplore.ieee.org/document/8456619). Please add those.

Reviewer #3 (Remarks to the Author):

Thank you for inviting me to review this manuscript. It is overall very well written and presents very nice images and results. It includes a lot of detail that will be useful for others working in this field. I recommend its publication but have some questions/comments that I think need to be addressed first.

My first impression was why this particular journal for this work, as it is not involving directly physics, I would classify it as biomedical engineering or biomedical imaging. Assuming the scope of the work has been established suitable for this journal, please see the following comments:
- I do not see any phase contrast in the paper – all results shown are normal (absorption-based) CT. Did I miss something? Surely if the title and main point is about phase contrast, this needs to be shown? Or at least discussed about how this will be done
- The setup is different from commercial small animal in vivo scanners in that the animal is in a vertical orientation, please explain how this is challenging to realize and how this may influence the animal, does it require more stringent/tighter “mounting” of the animal? I see some of this is mentioned in the methods at the end, but more information would be helpful.
- Related to this, can you please explain how the setup is different from small animal CT machines with gantry style acquisition? For example one commercial one allows up to 4 um isotropic voxel size according to specification sheet, see here for example: https://www.scanco.ch/vivact80.html . I would like to see a discussion of how the current setup with special X-ray source and phase contrast allows some benefit compared to this commercial style instrument

Great work overall, well done

Dear Editor and Reviewers,

We thank you for providing constructive feedback and thoughtful comments, which we used to improve our manuscript. Below is our point-by-point response (blue) including additions to the revised manuscript (red) according to the reviewer comments (black). Line numbers referred to in this response relate to the new revised manuscript.

Yours sincerely,
The authors

Reviewer #1

The manuscript presents three-dimensional in-vivo x-ray imaging of the lung from a free-breathing mouse using laboratory equipment (not a synchrotron radiation source), which is a great achievement. The manuscript is well written, clearly structured and the authors' conclusions are supported by the presented results. However, several important aspects are either not clearly stated or missing in the current manuscript and should be addressed in a minor revision before publication.

Response: We thank the reviewer for the overall recommendation.

1. The authors mention that the resolution is limited by the detector and the source size. It is not entirely clear to me, whether the blur from the source and the detector were matched. If the detector limits the resolution, one could probably slightly reduce the magnification accordingly, or in case the source is limiting the resolution, one could measure with a binning factor to achieve better statistics. This should be clarified in the manuscript.

Response: The source size has an elliptical shape which means that it is not possible to match it perfectly with the circular shape of the detector PSF. In the object plane the detector PSF is 80 µm / 6 (magnification) ≈ 13 µm which is in between the horizontal and vertical source size in the object plane (≈ 8.5 µm and ≈ 18 µm respectively), as stated in Lines 77-78 (paragraph on spatial resolution). As we prioritized the axial (horizontal) resolution for tomographic application (as stated in Lines 79-80), a lower magnification would yield worse spatial resolution due to increased effective detector PSF in the object plane, which is the limiting factor here. Theoretically due to the worse vertical resolution (which is source limited) we could perhaps perform 2x vertical binning (2 x 8.25 µm pixels = 16.5 µm) without a significant loss of axial resolution. This could be part of a future approach for reducing exposure times while preserving statistics.

To elaborate on binning as a future approach, we added the following sentence in the Discussion section:

Line 165: "Another way of reducing exposure times is to exploit binning in cases where the reconstructed voxels are fewfold smaller than the expected spatial resolution."

2. A Xineos-2329 mammography CMOS flat panel detector (Teledyne DALSA, CA) was used for the measurements. What sensor material does this detector use and how thick is the sensor? This is interesting to be able to judge on the dose efficiency of the presented results. Also, it would be very interesting to see a histogram of a raw projection to see how many counts the detector pixels collect during the 100 ms exposure. Are the images taken at the lowest limit of the dynamic range of this detector? What is the x-ray flux at these settings? What improvement in dose efficiency can be expected when using a photon-counting detector? Would the resolution still be high enough to resolve the edge enhancement for the phase-contrast imaging?

Response: The scintillator material is CsI, with the exact thickness kept secret by the manufacturer, but we expect it to be around 200-300 µm. A histogram for a 100 ms exposure is added as a new panel to Fig. 1 (d), see below. The dynamic range of the detector corresponds to 14 bit (i.e., maximum 16383 pixel value before saturation) which means that our projection images use only the low-portion of the detector dynamic range, as seen in the histogram. We estimate the flux per unit area on the detector to be ~3.5e5 X-ray photons/s/mm^2 (within the 15-30 keV spectral range which gives the main contrast here). Switching to a photon counting detector with similar pixel size should give a significant improvement in dose efficiency in many ways. Firstly the dark counts would be eliminated (cf. histogram in Fig. 1 (d)), and secondly the quantum efficiency should improve significantly resulting in much better statistics. Upcoming detectors (e.g. from Direct Conversion or Dectris) could provide imaging with similar spatial resolution, but are today still very expensive to manufacture in large pixel matrices (e.g., 4000x4000 pixels or more).

To include the detector scintillator and estimates of X-ray flux, we added the following sentences in Methods:

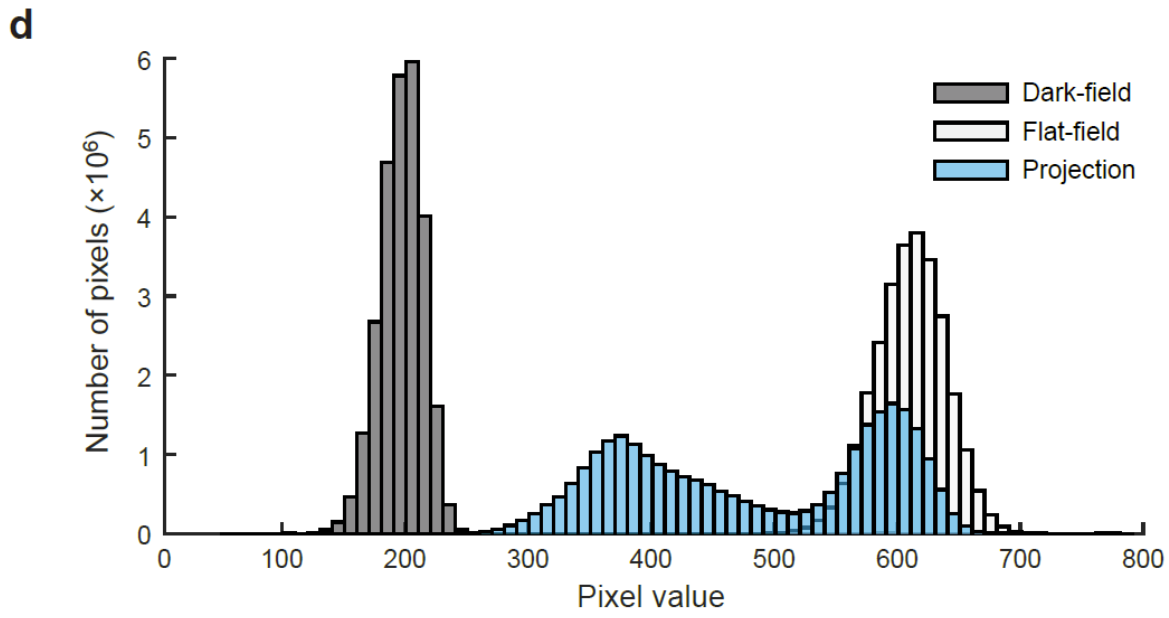
Line 216: “The scintillator material of the detector is CsI with a non-specified thickness but most likely 200-300 µm.”

Line 211: “The resulting X-ray flux on the detector plane is estimated to ~3.5e5 X-ray photons/s/mm^2 (within the 15-30 keV spectral range). This corresponds to ~90 X-ray photons (15-30 keV) per detector pixel during each 100 ms projection.”

Regarding the future use of photon-counting detectors we modified the section on the use improved detectors in Discussion:

Line 166: “Improved detectors based on photon-counting technology are likely to play an important role in keeping doses at acceptable levels by eliminating dark-counts (cf. Fig. 1d), read-out noise and improving quantum efficiency. The resolution of available photon-counting detectors should be high enough to resolve the edge-enhancement from the phase-contrast, but large pixel matrices are still expensive to manufacture as of today.”

Lastly we added a new panel (d) to Figure 1 with histograms of the pixel values in representative dark-field, flat-field and mouse projection images at a 100 ms exposure (with the corresponding caption):



Caption: "**d**, Histograms of pixel values in representative dark-, flat-field and mouse projection acquired with the detector during a 100 ms exposure."

We also added a sentence in the Results section regarding the dynamic range:

Line 56: "We note that our shortest exposure imaging already operates at the lower-end of the detector dynamic range (cf. Fig. 1d)."

3. The detector has a huge number of pixels (4608 x 5890 pixels). It is not absolutely clear from the manuscript, whether the projections were binned or not? By taking only 720 projections of the full detector image, the undersampling would be enormous. How many pixels on the detector are covered by the magnified sample?

Response: The projection images shown in the manuscript were not binned. Binning was only used as a part of the 3D segmentation pipeline, as stated in Line 265. The reviewer is correct in stating that 720 projections is certainly an undersampling. This is mainly a dose-related limitation (stated in Line 89) as radiation dose scales linearly with angular sampling. At a fixed dose, we prioritized statistics in each projection image (i.e. longer exposures) over a higher angular sampling in order to resolve the edge-enhancement from phase-contrast in each projection. For our application, the main factor in resolution degradation is still cardiac and residual respiratory motion, which means that increasing angular sampling will lead to higher dose possibly without increasing the resolution. The magnified mouse typically covers 3400-3800 pixels, so not the full detector area (as seen in the example projection image of the mouse in Fig. 1a).

To clarify the points raised above, we added the following to the Methods section of the revised manuscript:

Line 215: "No pixel binning was used for the imaging experiments."

Line 219: “The angular sampling used here is primarily limited by the suitable radiation dose per imaging session, but should otherwise be considered undersampled according to classical criteria (e.g., the Crowther criterion).”

4. Were the projections of the resolution phantom shown in figure 3a also phase retrieved using the Paganin algorithm? Without the phase retrieval the comparison with the reconstructed slide would not be fair, as the edge enhancement would most likely improve the resolution in the projections. This should be clarified in the text.

Response: The projection image of the phantom shown in Fig. 3a is not phase retrieved. The purpose of the projection image was to show the maximum 2D spatial resolution (therefore not phase-retrieved), while the tomographic slice (which was phase-retrieved prior to reconstruction) serves as demonstrating the tomographic resolution to be expected in a realistic in vivo tomography of a mouse. In other words, we wanted to show the two different aspects of our system (2D and 3D imaging), and not as a fair comparison.

To clarify, we modified the caption of Fig 3a (see additions in bold):

“[...] **a**, 2 s exposure projection image **(dark- and flat-field corrected but not phase-retrieved)** [...] **d**, Tomographic slice corresponding to a 300 mGy scan (100 ms exposure **projections, which were dark- and flat-field corrected and phase-retrieved**).”

Reviewer #2

In the manuscript “Phase-contrast X-ray tomography resolves the terminal bronchioles in free-breathing mice” the authors describe some proof-of-principle 3D imaging results of the tracheobronchial tree in free-breathing mice without mechanical ventilation. The results are very interesting for a broader biomedical community as they have been obtained at radiation levels compatible with longitudinal studies (i.e. > 500 mGy), and also within a small scale laboratory (in contrast to previous research at large-scale SR facilities).

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While my overall impression is therefore very positive (I highly recommend publication of this manuscript), I have a few smaller (optional) points that the authors might want to consider:

Response: We thank the reviewer for the positive recommendation.

1) Discussion of clinical impact: I fully agree that resolving the tracheobronchial tree down to the terminal bronchioles will enable very important biomedical research studies to better

understand diseases progression/treatment/etc. in connection with some lung diseases. It might, however be better suited for those diseases that have more impact on the bronchioles, rather than diseases that mainly/first affect the alveoli (which cannot be resolved at the moment). Good examples here might be small-airway disease, lung fibrosis, or actually COVID. Not so good examples are COPD and actually mechanical ventilation induced injuries (which mainly start in the alveoli). Please add a few sentences to the MS that discuss these potential clinical applications and for what it might best be suited at first.

Response: Thank you for the valuable insight. We have given this some thought and revised the the section on applications for lung-diseases in Discussion accordingly:

Line 137: "[...] respiratory disease in mouse models, primarily those affecting the bronchioles (e.g., asthma, bronchitis, small airway disease, COVID-19). Even though we cannot resolve individual alveoli yet, studies of diseases presenting alveolar abnormalities as early manifestations (e.g., COPD, pneumonia) could benefit from the improved spatial resolution demonstrated here. Abnormalities on the alveolar level should result in macroscopic structural changes to the parenchyma which could be studied in greater detail with our proposed arrangement."

2) Please discuss in more detail that the heart beat still degrades resolution and that this is worse the closer you get to the heart. Fig. 4a shows exactly that, and if the authors would have chosen a zoom in closer to the heart, this would also look a little more blurred. Just be clear on what is possible and what not (in 3D).

Response: To explicitly mention the effects of cardiac movement on image blurring we added the following sentence to Results:

Line 108: "The degradation of spatial resolution from uncompensated cardiac movement increases closer to the heart (cf. A Fig. 4a), which limits the resolvability of the nearby smallest airways."

3) In the introductory part, probably the best manuscripts to link to darkfield imaging might be Schleede et al, PNAS, 2012 (https://www.pnas.org/content/109/44/17880) and Gradl et al. IEEE TMI (https://ieeexplore.ieee.org/document/8456619). Please add those.

Response: The IEEE TMI article was already cited in the Introduction of the original manuscript (cf. [4]), but we agree that the PNAS article provides a relevant reference. We therefore replaced Ref. [3] with the suggestion from the reviewer.

We also take this opportunity to replace Ref. [6] (which was a preprint) with a recently published paper (during the review of this manuscript) from the same group demonstrating clinical dark-field imaging (https://doi.org/10.1148/radiol.2021210963).

Reviewer #3

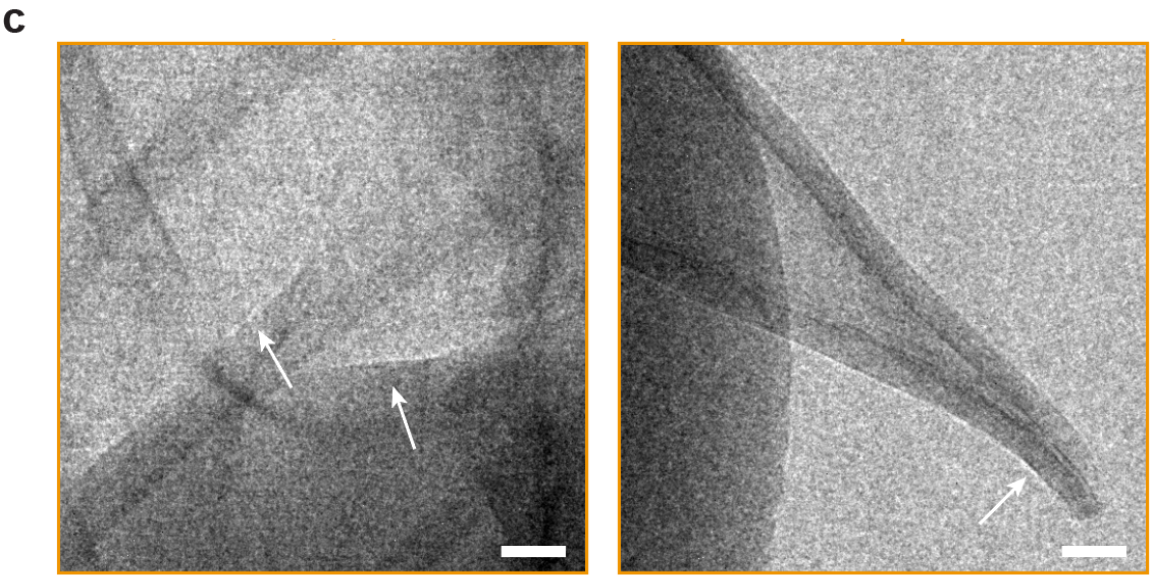
Thank you for inviting me to review this manuscript. It is overall very well written and presents very nice images and results. It includes a lot of detail that will be useful for others

working in this field. I recommend its publication but have some questions/comments that I think need to be addressed first.

Response: We thank the reviewer for the recommendation.

My first impression was why this particular journal for this work, as it is not involving directly physics, I would classify it as biomedical engineering or biomedical imaging. Assuming the scope of the work has been established suitable for this journal, please see the following comments:
- I do not see any phase contrast in the paper – all results shown are normal (absorption-based) CT. Did I miss something? Surely if the title and main point is about phase contrast, this needs to be shown? Or at least discussed about how this will be done

Response: The reviewer is correct that we do not explicitly show any phase-contrast edge-enhancement in our images. To improve the manuscript on this point, we added a new panel (c) to Fig. 1 which aims to demonstrate the effect of phase-contrast as subtle (bright) edge-enhancements of features in our projection images (with the corresponding caption):



Caption: "**c**, Zoom-in of the diaphragm (left) and ear (right) of a mouse captured in a 100 ms exposure projection (dark- and flat-field corrected). The phase-contrast produces edge-enhancement, seen here as a subtle bright contour around features (cf. arrows). Scale bars: 500 µm"

Prior to tomographic reconstruction (CT), the projection images are phase-retrieved (as explained in Methods, Lines 248 and onwards) which essentially converts the edge-enhancement seen above into an improvement of the conventional absorption contrast. Apart from increasing the visualization of air/tissue interfaces, most of the contrast in the mouse is still, as the reviewer suggests, based on absorption.

- The setup is different from commercial small animal in vivo scanners in that the animal is in a vertical orientation, please explain how this is challenging to realize and how this may

influence the animal, does it require more stringent/tighter “mounting” of the animal? I see some of this is mentioned in the methods at the end, but more information would be helpful.

Response: Vertical mounting has been used in a wide-range of imaging methods and is generally not a problem regarding animal well-being if the duration is short. From an imaging perspective, gravity can cause the mouse to move down during the acquisition resulting in image artifacts. Therefore vertical mounting requires a tight-enough restraining (wrapping with bandage as we did, or similar ways) to ensure minimal vertical movement during the scan, while still allowing for adequate respiration. From our experience this is not a major problem, and the restraining can be achieved with different approaches.

To clarify the importance of preventing vertical movement due to gravity, we added the following sentence in the Methods section:

Line 198: “It is especially important to prevent image artifacts arising from vertical movement due to gravity during the course of the tomographic acquisition. We achieved this through our tight bandage wrapping, but other approaches could yield similar results.”

- Related to this, can you please explain how the setup is different from small animal CT machines with gantry style acquisition? For example one commercial one allows up to 4 um isotropic voxel size according to specification sheet, see here for example: https://www.scanco.ch/vivact80.html . I would like to see a discussion of how the current setup with special X-ray source and phase contrast allows some benefit compared to this commercial style instrument

Response: Firstly, we want to stress that typically commercial systems specify achievable voxel-sizes, but rarely achievable resolution for a given task. Also, many commercial systems can resolve structures such as bones to high spatial resolution, but the contrast is not sufficient for visualising lung structures at the same resolution. To our knowledge, no commercial system available today can resolve airways below, say, 100 µm. Our system uses phase-contrast which improves contrast of air/tissue interfaces which is made possible using the most powerful microfocus X-ray source technology available today, which is typically not integrated in commercial systems.

To add to the discussion comparing our system to commercial ones, we added the following in Discussion:

Line 148: “Contrary to commercial preclinical micro-CT scanners which are often designed for fast low-dose scans and high-throughput studies, we designed our system for maximum spatial resolution and contrast in the lungs by using high-power liquid-metal-jet source technology enabling the long propagation distances required for phase-contrast imaging.”

Great work overall, well done

Response: Thank you.

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

The authors carefully addressed my questions and those raised by other reviewers. The manuscript improved after revision, especially the discussion section. I recommend the manuscript for publication.

Reviewer #2 (Remarks to the Author):

I am very pleased that the authors have followed up on all my suggestions for improvements. From my side all points have been addressed sufficiently, and thus I can fully recommend publication as is.

Reviewer #3 (Remarks to the Author):

All questions have been addressed, the manuscript can be accepted