3d Virtual Pathohistology of Lung Tissue from Covid-19 Patients based on Phase Contrast X-ray Tomography

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We present a new approach of three-dimensional (3d) virtual histology and histopathology based on multi-scale phase contrast x-ray tomography, and use this to investigate the parenchymal architecture of unstained lung tissue from patients who succumbed to Covid-19. Based on this first proof-of-concept study, we propose multi-scale phase contrast x-ray tomography as a novel tool to unravel the pathophysiology of Covid-19, extending conventional histology by a third dimension and allowing for full quantification of tissue remodeling. By combining parallel and cone beam geometry, autopsy samples with a maximum cross section of 4 mm are scanned and reconstructed at a resolution and image quality which allows for the segmentation of individual cells. Using the zoom capability of the cone beam geometry, regions-of-interest are reconstructed with a minimum voxel size of 167 nm. We exemplify the capability of this approach by 3d visualisation of the diffuse alveolar damage with its prominent hyaline membrane formation, by mapping the 3d distribution and density of lymphocytes infiltrating the tissue, and by providing histograms of characteristic distances from tissue interior to the closest air compartment.

1. Introduction

Severe progression of the 2019 coronavirus disease (Covid-19) is frequently accompanied by the clinical acute respiratory distress syndrome (ARDS) and respiratory failure, an organ manifestation responsible for the majority of Covid-19 fatalities. Lung injury associated with ARDS can be readily detected by radiographic chest imaging and clinical computed tomography (CT), which have assisted the diagnosis and management of intracellular virions and inflammation, disrupted cellular membranes, as well as widespread thrombosis with microangiopathy. As reported in [1], alveolar capillary microthrombi were found to be 9 times as prevalent in patients with Covid-19 as in patients with the also very aggressive H1N1 influenza A virus, also referred to as swine flu. Importantly, in Covid-19 lungs, a specific variant of new vessel growth - intussusceptive angiogenesis - was significantly more prevalent, i.e. 2.7 times as high as in lungs of patients with H1N1 influenza A.

Histomorphological assessment of formalin-fixed, paraffin-embedded (FFPE) tissue stained with haematoxylin and eosin still represents the gold standard in histological diagnostics of non-neoplastic lung diseases, including diffuse alveolar damage and virus induced pneumonia. In order to unravel the corresponding pathophysiology of the lung, digitalisation, visualisation and quantification of the morphological changes associated with Covid-19 represent a key challenge, and require both high resolution and the capability to screen larger volumes. For this reason, imaging the intricate three-dimensional (3d) tissue architecture of the lung and its pathological alterations on multiple length scales calls for 3d extensions of well-established histology techniques.

In this work, we use propagation-based phase contrast x-ray tomography as a novel tool for virtual 3d histology. We present first results obtained from postmortem lung samples of six patients who succumbed from Covid-19. We exemplify the capability of this approach by 3d visualization of the diffuse alveolar damage with hyaline membrane formation, by mapping the 3d distribution and density of angiocentric inflammation (perivascular T-cell infiltration), and by providing histograms of characteristic distances from the tissue interior to the closest air compartment.

Propagation-based x-ray phase-contrast tomography (PC-CT) has been introduced before for 3d virtual histology [3,6,7,8]. In contrast to conventional histology based on thin sections, it offers a full 3d visualization with isotropic resolution and without destructive slicing of the specimen. The interaction of x-rays with the object is described by the continuous complex-value index of refraction \( n(r) = 1 - \delta(r) + i\beta(r) \). Phase contrast capitalizes on the fact that for hard x-rays, the phase-contrast index \( \delta \) is several orders of magnitude higher in soft biological tissues than \( \beta \) which accounts for absorption

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Contrast is formed by transformation of the phase shifts into measurable intensity variations by self-interference of the exit wave during free-space propagation between sample and detector [10][11]. By careful optimization of photon energy, illumination function, and phase retrieval algorithms, the phase sensitivity is high enough to probe the small electron density variations of unstained tissue, for example tissue embedded in paraffin, ethanol, or even aqueous buffer [12]. In contrast to other full-field phase contrast techniques, e.g. based on grating interferometry or analyzer crystals, propagation based imaging (PBI) can reach a resolution below optical microscopy [13].

The 3d virtual pathohistology approach for Covid-19 presented here was realized by implementing a novel multi-scale phase contrast x-ray tomography concept, with dedicated x-ray optics and instrumentation to image the tissue structure on multiple length scales, while at the same time covering large reconstruction volumes. To this end, overview and regions-of-interest (ROI) scans were recorded on the same paraffin-embedded sample, covering a maximum tissue cross section of 4 mm by stitching different tomograms, and with a minimum voxel size of 167 nm in certain ROIs. Scale-bridging and dynamic ROI selection in close spatial and temporal proximity was implemented with dedicated instrumentation the GINIX endstation of the beamline P10/PETRA III (DESY, Hamburg) [14]. Specifically, we combined two optical geometries, which has only been realized at different synchrotron beamlines before: (i) Parallel beam tomography, covering a large field of view, with a pixel size of 650 nm. In this setting, a volumetric throughput on the order of $10^7 \text{ mm}^3$ was achieved, while maintaining the ability to segment isolated cells in unstained tissue. (ii) Cone beam geometry for recording of highly magnified holograms, based on advanced x-ray waveguide optics, providing mode filtering, i.e. enhanced spatial coherence and smooth wavefronts. Based on the geometrical magnification, the effective pixel size can be adjusted in the range 10–300 nm. The two imaging schemes are shown schematically in Fig. 1(c) and (d), respectively. Further, using this particular optics, together with appropriate choices of photon energy and geometric parameters, we can reach extremely small Fresnel numbers $F$ of the deeply holographic regime, well below the typical range exploited at other nano-tomography instruments. This offers the advantage of highest phase sensitivity sufficient to even probe the small electron density variations of unstained tissue, at relatively low dose [15]. To exploit this sensitivity, we use advanced phase retrieval methods including non-linear generalizations of the CTF-method [11] based on Tikhonov regularisation [16].

### Table 1: Sample and medical information

| sample no. | age (yr) | group, gender | hospitalization, clinical, radiological information | and histological characteristics |
|------------|---------|---------------|-------------------------------------------------|--------------------------------|
| I          | 60-70   | F             | 5-10d, GGO, DAD                                  |                               |
| II         | 80-90   | M             | 5-10d, DAD                                      |                               |
| III        | 90-100  | M             | 1-4d, GGO, DAD                                  |                               |
| IV         | 70-80   | M             | 1-4d, GGO, DAD                                  |                               |
| V          | 60-70   | M             | 5-10d, GGO, DAD                                  |                               |
| VI         | 70-80   | M             | 1-5d, GGO, DAD                                  |                               |
| CTRL       | 20-30   | M             | -                                                |                               |

2. Methods

2.1. Autopsy, clinical background and tissue preparation

In total we investigated six post mortem lung samples from Covid-19 patients [17]. A tissue micro-array paraffin block with samples of all six patients and the corresponding HE stain is shown in Fig. 1(b).

Information about age, gender, hospitalization, clinical, radiological and histological characteristics of all patients are shown in Tab. 1. All patients suffered from hypertension and were treated with RAAS (renin-angiotensin-aldosterone-system) interacting drugs. Heterogeneous ground glass and consolidation were observed in all patients clinical CT scans and the cause of death was also related to respiratory failure in each patient (patient IV cardio-respiratory failure). Additionally, a tumor-free lung samples from partial resections of pulmonary carcinomas were analysed as a reference.

From each of the six Covid-19 patients, two tissue samples with edge lengths of about 4 mm each were dehydrated. To one sample of each patient, a metal containing stain (uranium acetate, UA) was applied, the other samples remained unstained. Separated for their stain, six tissue samples were dehydrated and embedded in the same multi-sample paraffin block. The size of the post-mortem tissue samples made available for the study varied between the different patients (I-VI), with maximum cross-section of about 4 mm after dehydration. From all six samples, biopsy punches were taken by either a 8 mm or a 3.5 mm punch, depending on the individual size. The punches were then transferred onto a holder for the parallel-beam local tomography acquisition, followed by a further reduction in size (after measurement of the entire sample) to a 1 mm biopsy punch, for further tomographic recordings. A sketch of the sample preparation is shown in Fig. 1(c). The control lungs were first mounted in Eppendorf tubes, and 1 mm biopsy punches were then transferred to polymide tubes similar to the paraffin-embedded ones, but scanned in fixative buffer solution.

2.2. Phase contrast tomography

For Covid-19 lung tissue, the scans were recorded at the GINIX endstation of the PETRA III storage ring (DESY, Hamburg). The projections were acquired at two different photon energies $E_{ph}$, 8 keV and 13.8 keV, using the first and third harmonic of the 5 m P10 undulator and a Si(111) channel-cut monochromator, respectively. Data is shown here only for 8 keV, which gave highest contrast for the unstained lung tissue. Two tomography configurations were combined to cover a larger range of length scales:

(1-parallel beam) Recordings with the unfocused quasi-parallel beam illumination and a high resolution microscope detection system, resulting in a field-of-view (FOV) of $1.6 \text{ mm} \times 1.4 \text{ mm}$ sampled at a pixel size of 650 nm.
Fig. 1: Multi-scale x-ray tomography setup. (a,b) Sample preparation and mounting. (c) Configuration for parallel-beam tomography. (d) Configuration for cone-beam holo-tomography.

(2–cone beam) Holographic recordings with the divergent and coherence filtered beam emanating from a compound focusing system composed of a Kirkpatrick-Baez (KB) mirrors system and an x-ray waveguide, resulting in a FOV of 0.4 mm × 0.35 mm sampled at a pixel size of about 167 nm (depending on exact geometry).

Table 2: Data acquisition parameters.

| Cone geometry | Parallel geometry |
|---------------|-------------------|
| FOV | 0.4 mm × 0.35 mm | 1.6 mm × 1.4 mm |
| Pixel size | 167 nm | 650 nm |
| z₀₁ | 125 mm | - |
| z₁₂ | 4975 mm | 10 mm - 100 mm |
| Regime | holographic | direct contrast |
| Rotation | start-stop | continuous |
| Exposure | 2 s | 0.035 s |
| Total exposure | ≃63 min | ≃75 s |
| Volumetric flowrate | 1.16 × 10⁴ µm³ s⁻¹ | 3.75 × 10⁷ µm³ s⁻¹ |

Both configurations were implemented side-by-side, using the same fully-motorized tomography stage and mounting, as detailed in [15]. A sketch of both configurations is shown in Fig. 1. First, parallel-beam overview scans were acquired of the entire tissue volume embedded in paraffin. To this end a 3.5 mm biopsy punch was taken from the multi-sample tissue block, and then scanned in a stitching-mode yielding a large overview reconstruction, composed of up to 20 individual tomograms (depending on tissue size). To increase image quality and avoid artifacts related to region-of-interest (ROI) or local tomography, a selected 1 mm punch was then taken from the already scanned larger tissue cylinder, and re-scanned, first in the parallel- and then the cone-beam geometry. Experimental and acquisition parameters used for the data shown are listed in Tab. 2.

The configuration for (1–parallel beam) is depicted in Fig. 1c). The high-resolution microscope detection system (Optique Peter, France) was based on a 50 µm thick LuAG:Ce scintillator imaged with a 10× magnifying microscope objective onto a sCMOS sensor (pco.edge 5.5, PCO, Germany), resulting in an effective pixel size of 0.65 µm. The high photon flux density allowed for image acquisition with continuous motor movement, short acquisition time of 35 ms per frame, and a framerate of 20 fps. Single-distance tomogram recordings with about 1500 projections, and flat images before and after the scan, took less than 2 min. For these scans, the focusing optics (KB-mirrors and waveguide) as well as the fastshutter (Cedrat technologies) were moved out of the beam, and beam size was adjusted by the upstream slit systems. To avoid detector saturation, the beam was attenuated by 4×25 µm single crystal silicon wafers.

The configuration for (2–cone beam) is depicted in Fig. 1d): The beam was focused by the KB-mirrors to about 300 nm × 300 nm. To further reduce the secondary source size, to increase coherence, and to achieve a smooth wavefront for holographic illumination, an x-ray waveguide formed by 1 mm long lithographic channels in silicon with a cross-section of about 100 nm × 100 nm was positioned in the focal plane of the KB-mirrors, resulting in an exit flux of 1–4 × 10⁹ photon/s (depending on alignment and storage ring), as measured with the single photon counting detector (Pilatus, Dectris). The sample was positioned at variable (defocus) distances behind the focus (waveguide exit), typically z₀₁ = 125 mm for the first distance. The geometrically magnified holograms were recorded by a fibre-coupled sCMOS sensor (Zyla HF 5.5 detector, Andor Technologies) with a customised 15 µm thick Gadox scintillator, and 6.5 µm pixel size. The detector position at about z₀₂ = 5100 mm behind the focus resulted in a magnification of about M = 41 (for the first defocus distance), and
hence an effective pixel size of 167 nm. 1442 projections were recorded, with a typical exposure time of 2 s per projection.

2.3. Phase retrieval, image reconstruction, and segmentation

Phase retrieval and reconstruction Phase retrieval was performed from dark and empty beam corrected holograms, using both linearised single step CTF-approach [11] [19], and non-linear generalisations of the CTF-method based on Tikhonov regularisation (NL-CTF), using our code package HoloToolbox as described and deposited [18]. Importantly, both CTF and NL-CTF implementations can be augmented by imposing support and range constraints, when needed. When available, projections recorded at several defocus distances were first aligned with sub-pixel accuracy and then used for multi-distance phase retrieval. For these purposes, the HoloToolbox provides auxiliary functions, which also help to refine the Fresnel number or to correct for drift in the illumination function. After phase reconstruction of all projections, the tomographic reconstruction was carried out with the MATLAB implemented iradon-function (Ram-Lak filter) for the parallel geometry and with the FDK-function of the Astra toolbox [20] [21] for the cone beam geometry. Hot pixel and detector sensitivity variations as well as strong upstream window materials, which persist after empty beam correction, can all result in ring artifacts in the tomographic reconstruction, in particular as these flaws can increase by phase retrieval. To correct for this, the extra information provided by 360° scans was used to mask out the corresponding pixels and replace them with values of the opposing projection. Stitching of reconstructions from different tomographic scans was performed using [22].

Image segmentation and quantification For each patient the stitched overview scans were analysed with regard to structural characteristics. The 3d-reconstructions were first binned (2 × 2 × 2), and the tissue was then segmented from the surrounding paraffin using the segmentation software Elastik [23], which was then further refined with MATLAB. In order to exclude single macrophages or detached tissue, only voxels connected to the tissue block were considered for the distance map. Further, the areas of the paraffin which represent air compartments were linked to the outside of the tissue block. Individual self-contained areas of paraffin (not connected to air) were excluded from the mask. Based on this segmentation, the distance to the nearest voxel containing oxygen was calculated for each tissue voxel. The tissue volume V is given by the sum of all voxels containing tissue. The surface area S is defined by all tissue voxels with a distance of 1 pixel to the air. From this information we calculated the specific surface

\[ S_V = S / V \]

with \( S / V \) surface area volume -ratio. Further a characteristic length

\[ L_c = V / S \cdot v_x \]

with \( v_x \) edge length of a voxel was determined for each sample. Additionally, the mean distance \( \langle d_{ox} \rangle \) from all tissue voxels to air and its standard deviation was calculated.

Hyaline membranes and capillary networks were extracted using semi-manual segmentation functions in Avizo (Thermo Fisher Scientific, USA). Beside hyaline membranes and the capillary network, different cell types can be readily identified based on the 3d reconstructions of the electron density. In particular, inflammatory cell subpopulations – i.e. macrophages and lymphocytes - can be distinguished. To this end, an automatic and parameter-controlled algorithm denoted as Blobfinder was used (Arivis). Based on its segmentation output, the amount and position of the lymphocytes in the 3d-reconstructions from parallel beam scans was calculated. The algorithm is able to identify roundish structures with a given size. For the segmentation of the lymphocytes, we chose a characteristic size of 6.5 μm. The structures identified in this step also include macrophages and parts of the capillary system. For the unbinned datasets, a distinction between lymphocytes and the nuclei of the macrophages was made based on the difference in electron density. In the tomographic reconstructions, the lymphocytes appear denser compared to the nuclei of the macrophages. Further, nuclei from endothelial cells and parts of the capillaries filled with blood residues were excluded based on their elongated shape. Only structures with a sphericity higher than 0.55 were included. Based on the segmentation of lymphocytes, the total number of lymphocytes \( N_l \) was obtained and the mean concentration of lymphocytes within the lung tissue \( c_l = N_l / V \) was estimated.

3. Results

Before presenting the results for the six Covid-19 patients for each of the imaging levels (scales), we give an overview on the typical datasets for one exemplary sample (I), see Fig. 2. The typical field-of-views, image quality, and appearance of the lung structure as well as the amount of data can be inferred, and inspected in the reconstructions provided online (https://doi.org/10.5281/zenodo.3892637). The datasets are denoted by patient I-VI, respectively. Gray values of the tomographic reconstruction represent phase shift per voxel with edge length \( v_x \), the local electron density difference to the average paraffin can be computed by

\[ \Delta \rho(r) = \frac{\varphi_{v_x}(r)}{v_x \cdot \lambda \cdot r_0}, \]

with wavelength \( \lambda \) and \( r_0 \) classical electron radius.

3.1. Reconstructed electron density

Next, we present representative slices through the reconstruction volumes of all samples for all acquisition scales. Conventional HE-stained histology images of all samples are shown in the supplementary information (Fig. [11]). Fig. 3 presents the stitched reconstruction volumes, recorded under conditions of local tomography, see Tab. 2. Since these volumes are computed from stitching up to 24 individual tomograms, the question arises to which extend the image quality is limited by potential artifacts of local tomography, i.e. errors due to the fact that part of the sample is outside the reconstruction volume. For this reason, 1 mm punches were taken after the stitched overview and rescanned in the parallel beam configuration, without local tomography conditions, since they fitted within the FOV. The results are presented in Fig. 4 and validate the previous stitching results. The 1 mm punches then also provided an appropriate size for the cone-beam recordings, which are shown in Fig. 5. Importantly, in each scan the previous level guided the choice for the next FOV
and informed about the larger environment. In the following, we briefly discuss the samples one-by-one, with regard to all acquisition scales. A comparison of morphological features between conventional and virtual histology is shown in the supplementary (Fig.12).

**SAMPLE I:** By conventional histopathological assessment, the peribronchial alveolar parenchyma of sample I showed DAD with focal formation of hyaline membranes adjacent to the epithelial lining, moderate lymphocytic interstitial pneumonia and singular thrombi in small pulmonary veins. There is a moderate hypertrophy of the muscular media in smaller pre- and post-capillary blood vessels with desquamation of the endothelial cell layer as well as mild centrilobular emphysema (original magnification 100x). In PC-CT, enlarged alveolar septa with pronounced lymphocytic inflammation are displayed. The reconstruction volume contains a large artery filled with erythrocytes (Fig. 3, lower left), which bifurcates into two vessels. This area was then selected for the 1 mm biopsy punch extraction. The cone-beam zoom tomogram was then centered around the perimeter of the blood vessel. This volume is particularly well suited to investigate the connective tissue including elastic fibers and collagen, as well as smooth muscle.

**SAMPLE II:** Histomorphological analysis shows peribronchial alveolar parenchyma with hyperemia of capillary and post-capillary blood vessels, as well as a moderate centrilobular emphysema (original magnification 100x). On the level of blood vessels, both blood-filled and empty vessels are discernible. It should be noted that septa with signs of parallel capillaries are visible. In the reconstruction volume of the zoom tomogram, a single vessel can be easily tracked over large distances.

**SAMPLE III:** The sample consists of peribronchial alveolar parenchyma showing prominent multifocal neutrophilic capillaritis as well as a moderate centrilobular emphysema (original magnification 100x). In PC-CT, septa with again similar physiological size and distribution emerge, with moderate emphysema. The bottom part of the sample contains a fibrous area near a larger blood vessel. The zoom tomogram shows a single septum, a blood vessel and a fibrous region.

**SAMPLE IV:** Histomorphological analysis shows peribronchial alveolar parenchyma with marked lymphocytic interstitial pneumonia, multifocal venous thrombi and focal intraalveolar fibrin deposition in terms of DAD. Furthermore, there is a mild centrilobular emphysema (original magnification 100x). In PC-CT, a network of thin septa, thrombi and emphysema, as well as a large empty blood vessel appears. Electron-rich diffuse black granules are also visible. The biopsy punch was selected to contain the empty blood vessel, some small thrombi. It also includes thin septa and tissue embedded dirt-particles. The zoom tomogram covered tissue with black granules as well as a band of inflammatory cells next to a...
Fig. 3: Stitched parallel-beam reconstructions for full pulmonary samples (I-VI). Representative virtual sections through the reconstruction volumes of full biopsies (I-VI), respectively. Scale bars: 500 µm.

Sample V: By conventional histological assessment, Sample V consists of peribronchial alveolar parenchyma showing massive lymphocytic interstitial pneumonia with ubiquitous hyaline membranes superimposed on the alveolar walls, neutrophilic capillaritis and multifocal post-capillary thrombi in terms of severe DAD. Furthermore, bronchialized alveolar epithelial cells show cytopathic changes and multifocal desquamation, as does alveolar macrophages. Focally, accumulation of intraalveolar neutrophilic granulocytes in the sense of a florid bronchopneumonia can be observed (original magnification 100x). PC-CT data give rise to alterations of the overall morphology due to Covid-19, including substantial inflammation, pronounced hyaline membranes, and high load of lymphocytes. The biopsy punch was chosen to include areas with increased presence of hyaline membranes and lymphocytes. A blood vessel splitting into several smaller blood vessels is easily recognized when browsing through the reconstructed volume. Noteworthy, different cell types as macrophages, T-cells or erythrocytes can be distinguished in the zoom tomogram.

Sample VI: Histomorphological analysis shows peribronchial alveolar parenchyma with lymphocytic interstitial pneumonitis and a singular thrombus in a small vein. The interstitium of the alveolar septae are widened by myogenic metaplasia. Adjacent, centrilobular emphysema and anthracosis are observed. The bronchial mucosa shows varying degrees of lymphocytic inflammation in the sense of chronic bronchitis / bronchiolitis (original magnification 100x). From PC-CT reconstructions, the sample consists of thin alveoli (Fig. VI, upper left) evolving into compact, fibrotic tissue (Fig. VI, lower right). The amount of lymphocytes is rather low in this sample. Black granules and some thrombi are embedded within the bulky tissue parts. The biopsy punch covers the region of transition from alveoli to fibrotic tissue, containing also a thrombus and capillaries as identified from the zoom tomogram.

3.2. Visualisation of pathologies, segmentation, and quantification

The overview scans of all paraffin embedded Covid-19 positive samples and one biopsy of a hydrated control lung were analyzed in terms of structural characteristics. The top row of Fig. 5 shows the workflow of the analysis on the example of the 3d reconstruction of sample V. Based on the tissue mask the distances for each tissue voxel to the next voxel containing oxygen were determined. For the analysis of the tissue it is mandatory to consider the three-dimensionality of the samples. This can also be seen in Fig. 6, which shows a zoom of the distance analysis around a small blood vessel which is marked with a yellow box. While the wall thickness appears quite homogeneous in the 2d slice, the distance analysis reveals that
Fig. 4: Parallel-beam reconstructions for 1 mm biopsy punches (I-VI). Representative virtual sections through the reconstruction volumes of the 1 mm punches into the volumes of the full biopsies (I-VI), respectively. The fact that the punches are isolated results in higher image quality, since the errors associated with local tomography are avoided. Scale bars: 100 μm.

Fig. 5: Cone beam reconstructions for biopsy punches (I-VI), shown for approximately the same slices as in Fig. 4 for the parallel beam reconstruction. Virtual sections through the reconstruction volumes of the cone-beam recordings corresponding to sections in Fig. 4 obtained by the parallel beam configuration, for biopsies (I-VI), respectively. Scale bars: 100 μm.
Fig. 6: Tissue compactness and distance metrics: The 3d reconstructions of the lung tissue were analysed in terms of specific surface and characteristic lengths. The workflow is sketched for sample V in the top part (a-g). In a first step, the tissue was segmented using Ilastik. A slice of the reconstructed electron density is shown in (a). Based on the segmentation, the areas of air which are directly connected and potentially filled with oxygen (or blood) are masked out (b). (c) For each of the remaining tissue voxels the shortest possible distance to air was calculated. Especially around vessels and larger alveoli, the distances are larger. (d) Zoom into an area around a vessel. Further analysis is based on the distance distribution shown in (e). (f) Volume rendering of the reconstructed electron density, with (g) showing the corresponding 3d distance map. (h) Based on the tissue segmentation of all samples, the distance from the tissue interior to the closest air compartment was calculated. In order to compare all samples, the count of voxels was normalized by the total volume of the respective sample. The specific surface area $S_V$ (represented by the first value of each curve), the characteristic length $L_c$ and the mean distance $d_{O_2}$ for each sample was calculated based on this data. Double logarithmic scale, bin width of the distribution of distances: 1 µm. Scale bars: (a-c) 100 µm.
the vessel is thicker on the top right. The distribution of the
distances obtained for a given slice is shown in Fig. 6f. The
swelling of the alveolar walls as well as the inflamed blood ves-
sels can be identified by comparing the reconstructed electron
density and the 3d-distance map (see Fig. 6f and g).

Fig. 6h shows the distribution of the tissue-air distances (histogram)
for all samples, following the workflow illustrated in Fig. 6i. The binning of distances was set to one voxel length. The figure underlines the high diversity of the tissue structure which could already be seen in the 3d histology. Further, it
directly informs about the specific surface $S_V$, which is given
by the first point of the graph.

Table 3: Results of the analysis of tissue characteristics: spe-
cific surface area $S_V$, characteristic length $L_c$ and mean dis-
tance $d_{G2}$ and standard deviation from all tissue voxels to air
as well as the mean concentration of lymphocytes $c_l$ for all six
Covid-19 positive samples as well as for one control sample.

| patient no. | $S_V$ (%) | $L_c$ (µm) | $d_{G2}$ (µm) | $c_l$ (1/mm$^3$) |
|-------------|-----------|------------|--------------|-----------------|
| I           | 13.87     | 9.4        | 5.9±3.5      | 16.0·10$^7$     |
| II          | 46.56     | 2.8        | 2.1±1.0      | 14.1·10$^5$     |
| III         | 33.75     | 3.9        | 2.5±1.5      | 4.4·10$^5$      |
| IV          | 25.90     | 5.0        | 3.2±2.3      | 7.1·10$^5$      |
| V           | 19.28     | 6.7        | 3.6±2.1      | 4.8·10$^5$      |
| VI          | 11.87     | 11.0       | 9.1±10       | 6.1·10$^5$      |
| CTRL (hyd.)| 20.04     | 6.5        | 5.0±5.1      | -               |

The corresponding parameters and metrics are tabulated in Tab. 3 for all samples. Additionally, the mean concentration of lymphocytes $c_l$ within the lung tissue is listed for all samples. The values quantify the general structure of the tissue which is qualitatively discernible by eye. Samples with a high amount of swollen, inflamed blood vessels and thick
hyaline membranes exhibit a larger characteristic length.
Note, that the control lung was prepared in a hydrated
environment and shrinking due to further preparation of the
sample does not occur. Hence, the results cannot be directly
compared to the paraffin embedded samples. Further, the
analysis of the lymphocyte concentration was be performed
since no lymphocytes were found in the reconstructed volume.
The low values of $L_c$ and $d_{G2}$ for sample II correlate with the
lack of ground-glass opacification in clinical CT. Based on the
extracted structural parameters, the degree of inflammation
and swelling of lung tissue can be evaluated. E.g. patient
II has the highest surface area volume-ratios while sample
I and VI have a relatively low specific surface. Larger
characteristic lengths may also be indicative of inflammation
and the formation of hyaline membranes, which will be
evaluated in the following based on ROI and high-resolution
reconstructions.

Fig. 7 illustrates the aggregation of hyaline membrane in
the vicinity of a single alveole. Volumetric renderings in (a)
and (b) demonstrate particular attachment of fibrin to the
alveolar walls. In cases of severe hyaline membrane formation
as for this patient, this pathological alteration can be tracked
throughout the volume (c-e). In (f), hyaline membranes of
neighboring alveoles are indicated. In the 3d-context, their
locations with respect to blood vessels can be inspected, see (g),
which exemplifies a direct connection of hyaline membranes
to the vasculature.

The severeness of hyaline membrane formation is case-
specific, as the yellow rendering in Fig. 8(a, b & d) demon-
strates reduced amounts of deposits for patient I in a subvol-
ume of parallel-beam reconstructions. Further, lymphocytes
(red) were identified based on the automated cell segmentation
(see Methods). For clearer visualisation, each cell is rendered
as a sphere with a size corresponding to the mean cell volume
in (a, b & d). Based on convoluation of the cell positions
with a sphere of 100 µm in radius, the local cell density was calculated (b) and presented as 3d-maps of cells/mm$^3$ in (c &
e). This concept was then translated to a 3d-stitched volume
of an entire tissue block as shown in (f & g).

Next, the segmentation of blood vessels is demonstrated for
the example of a splitting blood vessel in the zoom tomogram
of sample V. The segmentation was performed manually. To
give an impression of the separation of a single capillary, a
series of virtual slices in the xy-plane (magnified views) is
shown in Fig. 9. The separation starts with the creation of
a branch from the blood vessel (arrow in slice 336). In slice
326, 1.7 µm above slice 336, this branch evolves into an empty
and separated capillary. Another 2 µm above, the capillary is
entirely filled with cells. Further 2.3 µm, the capillary is empty
again and has a diameter of about 2.8 µm. The segmentation
of the blood vessel with all its separated branches is indicated
for slice 336 in Fig. 9b by the red lines. In this slice, three
capillaries have already separated from the main vessel, while
the fourth starts to emerge, indicated by the red arrow. The 3d
shape of the blood vessel is illustrated by the 3d rendering of
the segmentation, shown in Fig. 9c. This segmentation of the
blood vessel shows the potential of the datasets, which may be
fully exploited in future with more advanced segmentations.

4. Summary, Conclusion and Outlook

In summary, we have demonstrated that multi-scale x-ray
phase contrast tomography can firstly augment pathohistol-
ogy of the lung to full three dimensions, and secondly provide a
link between microscopic and macroscopic scales. For the first
time, characteristic morphological changes associated with
diffuse alveolar damage such as hyaline membranes and pro-
nounced inflammation have been imaged in three dimensions,
in particular in Covid-19, the most severe pandemic which
mankind has faced for decades. Moreover, we conducted our
study in FFPE material, a fixation method established for
well over a century and ubiquitously available.

On the side of imaging technology and exploitation, there
is still ample room for improvement: higher resolution could
be achieved by further geometrical zooms, waveguide optics
with higher numerical aperture, in combination with pixel
detector technology, and further improvements in holographic
reconstruction. Equally important to extend the length scales
covered to small scales is the further upscaling of the field of
view (FOV) based on stitching different sub-tomograms. In
this way, one could bridge scales to cover the entire lobe of a
lung. To this end, optimized recording and data flow is a larger
bottleneck than photons and optics, in particular since 4th
generation synchrotron radiation sources are about to deliver
unprecedented brilliance. Finally, in view of exploitation and
information gain, specific labels for different cell types and
3d immunostaining coupled to radiocontrast agents should be
developed. Here, lung offers an advantage over other tissue in
view of volume accessibility and label diffusivity.
Fig. 7: Rendering of hyaline membrane attached to alveolar walls (patient V, parallel beam-scan of a 1 mm punch).

The rendered subvolume was restricted to $1.15 \times 1.10 \times 0.56 \text{mm}^3$, to contain a single alveole foremost. (a) Volume rendering of the segmented hyaline membrane in same spatial orientation as (c)-(e), which show virtual slices through the (c) top, (d) center and (e) bottom of the alveole. For a better spatial classification, (b) gives a combination of the volume in (a) and the slice in (d). (f) Volume rendering of the entire subvolume including neighbouring alveolae. (g) Zoom-in onto a major blood vessel (red) which is directly connected with the hyaline membrane. Scale bars: (c-e) 300 $\mu$m.

Most importantly, efforts have to be directed to exploit the full information contained in the reconstruction volumes based on advanced segmentation. Tracing of capillaries are an obvious important next step, in view of unraveling the role of intussusceptive angiogenesis in Covid-19. The current data, which is made fully available at https://doi.org/10.5281/zenodo.3892637, is likely to already provide such clues once that more advanced segmentation approaches based on machine learning are applied. More generally, it may advance our understanding of diffuse alveolar damage in the particular case of Covid-19, as an intrinsically volumetric phenomenon. Beyond the current data, further studies could shed further light on the differences between moderate and severe progression. Possibly, phase contrast tomography of lung biopsies could in future also help diagnosis and treatment.

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Competing Interests

The authors declare no competing interests.

Ethics

The study was approved by and conducted according to requirements of the ethics committees at the Hannover Medical School (vote Nr. 9022 BO K 2020).

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Fig. 8: Rendering of alveolar wall with hyaline membrane and quantification of lymphocyte infiltration (unstained tissue, patient I, PB-scan of a 1 mm punch). The illustrations in (a-e) show a subvolume of $0.60 \times 0.48 \times 0.26 \text{ mm}^3$, (f & g) this concept applied to the full tissue block of $2.57 \times 2.99 \times 0.98 \text{ mm}^3$. (a) Yellow contours mark the locations of hyaline membrane in an exemplarly slice. In same spatial orientation, (b, d & f) volume rendering of soft tissue (light pink), infiltrated by hyaline membrane (yellow) and lymphocytes (red) and (c, e & g) their local cell density among the lung tissue, including air compartments.
Fig. 9: Segmentation of the blood vessel network, exemplified for biopsy (V). (a) Series of slices in z-direction illustrate the separation of a capillary (≈ 5 µm diameter) from a blood vessel. Red arrow marks the separating capillary. In slice 314, the capillary is entirely filled with erythrocytes. (b) Red lines in the entire virtual slice (336) represent the contours of a manual segmentation of the blood vessel network. Three capillaries already separated from the main vessel, while the fourth starts to emerge, indicated by the red arrow. (c) Segmentation illustrating the 3d structure of the blood vessel network. Scale bars: 50 µm.
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A. Supplementary Information: Methods & Datasets

The imaging workflow may also be applied to hydrated and/or healthy lung tissue for systematic pathological analysis, as indicated in Fig. 10. (a) shows the rendering of truncated hydrated control tissue volume. (b) and (c) show virtual slices through the volume, demonstrating the parenchymal architecture in unaffected lung autopsies.

A.1. Medical Information and Correlative Histology

Before taking tomograms of the samples at the synchrotron setup, tissue slices of 2.5 µm thickness were cut from the top, afterwards HE (hematoxylin and eosin) stained and imaged with a microscope. Figure 11 shows the histological slice of each sample.

A comparison of morphological features between conventional HE histology and virtual histology is presented in Fig. 12. Artery lumen, artery wall, erythrocytes, thrombus, alveolar septum, macrophage, hyaline membrane and black granules (anthracosis) are shown for both imaging methods. Contrast of the hyaline membrane is homogenous in both histologies, making it simple to recognize and segment. Erythrocytes are easy recognizable by eye in the conventional histology, due to the HE staining, while they are less eye catching in the virtual histology. This results in a difficult differentiation between thrombus and blood stasis as well as a difficult identification of blood capillaries in the alveolar septum in virtual histology.

A.2. Further datasets

For each of the six unstained and FFPE tissue samples, there is also a UA-stained block sample of similar size. Stitched overview scans in (1 - parallel beam) configuration have been recorded, analogue to Fig. 3. From the UA-labelled tissue blocks of patients I, III, IV and V, 1 mm biopsy punches have been inspected in the same configuration (cf. Fig. 4). Using the (2 - cone beam) setup, these samples from I, III and IV were imaged at 8.0 keV x-rays, while V has been examined at 13.8 keV, as shown for Fig. 5.

Further, scans of the unstained tissue block from patient II have been performed at different propagation distances (z_{12} = 50, 100 and 125 keV) and different x-ray energies (13.3, 13.8, 14.3 and 14.8 keV). In cone-beam configuration, the unstained biopsy punch from patient I was scanned using 13.8 keV x-rays.

From all healthy, hydrated tissue blocks, overview scans covering the entire samples have been recorded. Hydrated 1 mm biopsy punches (two for CTRLI, one for CTLRII & CTRLIII, where CTRLII & CTRLIII have been extracted from the same patient) have also been recorded in (1 - parallel beam) configuration. Those from CTRLII & CTRLIII were also examined in (2 - cone beam) mode.

A.3. Method translation to compact µCT instrumentation

Prior to the synchrotron experiment, some of the samples have been examined with a laboratory phase-contrast µCT-setup in large mm²-sized FOV-configuration (Kα = 9.5 keV, \( \mu_{\text{eff}} = 5 \mu m \), \( z_{12} = 1.7 m \), 1200 projections of 1 s exposure time with a flat panel CMOS detector with 150 µm Gadodextran-scintillator, PerkinElmer, USA) [24]. UA-staining of the tissue proved advantageous in feature contrasting when working with laboratory x-ray sources. Aiming at overview data with no claim for refined resolution, structures can be well-correlated with histological sections in Fig. 13.
Fig. 10: Illustrations of control lung tissue (hydrated). (a) Volume rendering of the tissue block (0.97 × 1.00 × 0.73 mm³) and (b) slice through the volume, examined in PB-configuration. (c) Slice from the cone-beam scan, arrows indicating the structure of a healthy septum. Particularly, macrophages and erythrocytes emerge. Scale bars: (b) 100 µm and (c) 300 µm.

Fig. 11: Microscopic images of HE-stained histological sections of all samples (I-VI). Histological slices show comparable morphologies to the virtual slices in Fig.3, which represent different z-position. Scale bars: 400 µm.
Fig. 12: Comparison of morphological features between conventional HE histology and virtual histology. Artery lumen, artery wall, erythrocytes, thrombus, alveolar septum, macrophage, hyaline membrane and anthracotic pigments (i.e. the black granules) are presented on exemplary slices of different samples (I-VI) for conventional (left) and virtual histology (right). Scale bars: left 200 µm, right 100 µm.
Fig. 13: First screening with a laboratory phase-contrast µCT-setup (UA-stained tissue block, patient I). (a) Histological and (b) correlative virtual slice from laboratory phase-contrast tomography. (c) Volume rendering of the entire tissue block from a similar perspective. Scale bar: 1 mm.