Application of ethyl cinnamate based optical tissue clearing and expansion microscopy combined with retrograde perfusion for 3D lung imaging

Quanchao Suna,b,c, Tiziana Picasciaa,c, Arif ul Maula Khan a,c, Cinzia Brennaa,c, Vincent Heuvelined, Anja Schmause,f, Jonathan P. Sleeman e,f and Norbert Gretza,c

aMedical Research Center, Medical Faculty Mannheim, University of Heidelberg, Mannheim, Germany; bDepartment of Thoracic Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China; cInstitute for Medical Technology, University of Heidelberg, and University of Applied Sciences, Mannheim, Germany; dDirector of the Computing Centre, Heidelberg University, Heidelberg, Germany; eEuropean Center for Angioscience (ECAS), Medical Faculty Mannheim, University of Heidelberg, CBTM, Mannheim, Germany; fInstitute of Biological and Chemical Systems–Biological Information Processing (IBCS-BIP), Karlsruhe Institute for Technology (KIT), Karlsruhe, Germany

ABSTRACT

Purpose: 3D imaging of the lung is not a trivial undertaking as during preparation the lung may collapse. Also serial sections and scans followed by 3D reconstruction may lead to artifacts. The present study aims to figure out the best way to perform 3D imaging in lung research.

Materials and Methods: We applied an optical tissue clearing (OTC) method, which uses ethyl cinnamate (ECi) as a fast, nontoxic and cheap clearing solvent, for 3D imaging of retrograde perfused lungs by laser confocal fluorescence microscopy and light sheet fluorescence microscopy. We also introduced expansion microscopy (ExM), a cutting-edge technique, in 3D imaging of lungs. We examined and compared the usefulness of these techniques for 3D lung imaging. The ExM protocol was further extended to paraffin-embedded lung metastases blocks.

Results: The MHI148-PEI labeled lung vasculature was visualized by retrograde perfusion combined with tracheal ligation and ECi based OTC. As compared with trans-cardiac perfusion, the retrograde perfusion results in a better maintenance of lung morphology. 3D structure of alveoli, vascular branches and cilia in lung were revealed by immunofluorescence staining after ExM. 3D distribution of microvasculature and neutrophil cells in 10 years old paraffin-embedded lung metastases were analyzed by ExM.

Conclusions: The retrograde perfusion combined with tracheal ligation technique could be applied in the lung research in mice. 3D structure of lung vasculature can be visualized by MHI148-PEI perfusion and ECi based OTC in an efficient way. ExM and immunofluorescence staining protocol is highly recommended to perform 3D imaging of fresh fixed lung as well as paraffin-embedded lung blocks.

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Introduction

Conventional histological methods can provide only two-dimensional (2D) planar images that lack the three-dimensional (3D) structures consisting of a variety of constituents.\(^1\) Serial sections and scans followed by 3D reconstruction can lead to a loss of detailed anatomical information of thick lung tissue.\(^2,3\) Therefore, 3D imaging of thick tissue will make a big difference to visualize complex structures in the lung, such as vasculature, cancer cell accumulation or immune cell distribution, which are difficult to investigate in 2D planar sections.

Optical tissue clearing (OTC) makes biological specimens transparent by reducing the refractive index (RI) difference within tissues to overcome limited penetration depth of light in sample, which enables 3D imaging.\(^4\) Various OTC methods have been developed to visualize all types of tissues. They can be divided into three main categories according to different working principles: (1) simple RI matching, such as Sucrose, SeeDB and Focusclear;\(^5,6\) (2) delipidation combined with dehydration (solvent-based) or hyperhydration (aqueous-based) followed by RI matching, including DISCO-based methods (iDISCO,
uDISCO, vDISCO and FDISCO) and CUBIC,8,9 and (3) tissue-gel hybridization followed by delipidation and RI matching, including CLARITY, ELAST, SHIELD and eFLASH.10–12 Recently, there is a growing interest in optimizing OTC protocols to better understand structural changes in different kinds of healthy and diseased tissues. The ECi based OTC protocol, which is characterized with cheap, nontoxic, ready-to-use solvent and efficient clearing process, was firstly reported by Klingberg et al in kidney research.13 The principle of ECi based OTC includes dehydration with different concentration of ethanol and refractive index matching to ECi (≈ 1.558). In the past two years, ECi based OTC is becoming more and more popular in different kinds of tissues apart from kidney. Our group optimized the primary ECi based OTC protocol in previous research. The whole dehydration and clearing process were performed under vacuum and at room temperature by an automatic tissue processor, which allows high throughput and is more efficient. The room temperature and vacuum can improve the diffusion of reagents into tissues. The samples were brought from 50% directly to 80% ethanol in the optimized protocol instead of from 30% to 70% ethanol in the original.14 These changes were adopted to ensure a faster and stronger dehydration. Furthermore, MHI148-PEI, a dye previously developed at the Medical Research Center of the Universitätsklinikum of Mannheim, Germany, is characterized with a large number of positive charges, infrared fluorescence emission and high molecular weight.14 The positive charges come from the branched PEI group and can promote the electrostatic binding of the dye and the glycosaminoglycans on the vessel wall. A fluorescent dye with infrared wavelength can always give better results due to their low signal to noise ratio and higher penetration rate into tissue. However, there is not any specific report about the application of ECi based OTC or MHI148-PEI in the histological morphology research of lung tissue until now.

In this study, we introduced ECi based OTC and MHI148-PEI-vessel labeling combined with retrograde perfusion via abdominal aorta in lung research. The novel ExM technique was applied to do 3D high-resolution imaging of lung tissue in mice. 3D structure of the lung tissue was visualized by these methods using Laser confocal fluorescence microscopy (LCFM) or Light sheet fluorescence microscopy (LSFM). The ExM protocol was further extended to explore the microvasculature and neutrophil cell distribution in 10 years old paraffin-embedded lung metastases blocks. Both the advantages and disadvantages of these protocols were analyzed to provide a guidance on how to figure out an appropriate way to do 3D imaging in lung research.

Materials and methods

Animals and metastases specimen

8 weeks old, specific pathogen free (SPF) female C57BL/6 mice (25–38 g) were kept in a temperature controlled SPF facility with a 12:12 h light:dark cycle and ad libitum access to food and water. All experimental procedures were approved by the local authority and conducted in accordance with all guidelines and regulations set by the Germany Animal Protection Law and EC directive 2010/63 EU. Paraffin-embedded lung metastases blocks were provided by Prof. Jonathan P. Sleeman. The lung metastases were induced by intravenous injection of Lewis lung...
cancer cells into BALB/c mice. Experiments were performed five times and repeated with similar results.

**Retrograde perfusion and lung extraction**

The perfusion device is made up of a series of tubes connected to a Jun-Air compressor with two bottles filled with saline/heparin (0.9% saline + heparin 5 IU/mL) and 4% PFA in 1× PBS. A syringe pump is connected to the export tube via a T-junction, which can be used for the MHI148-PEI staining (Figure 1A). The mice were deeply anesthetized by intraperitoneal injection of ketamine (120 mg/kg BW)/xylazine (16 mg/kg BW). The whole body perfusion was performed by inserting a needle into the bifurcation of the abdominal aorta (Figure 1B), which results in a retrograde perfusion (Figure 1E, Perfusion). To avoid squeezing lung, we always performed thoracotomy and lung extraction after the cervical trachea is isolated and ligated with a suture. In order to avoid that long-term fixation and preservation worse autofluorescence and quench specific fluorescence, lungs were post fixed in 4% PFA overnight at 4°C in the experiment.

**ECi based OTC**

The dehydration and ECi clearing were performed in a Leica tissue processor (TP1020) automatically at room temperature. Briefly, 4% PFA fixed lungs were dehydrated by increasing concentrations of ethanol (50%, 80%, 100%, 100%), each step lasting 30 min, and then they were cleared in ECi for 2 hours (Figure 1E, ECi-OTC). Finally, the specimens were moved into black tubes filled with ECi and stored at room temperature on a rotary.

**ExM and 3D immunofluorescence staining**

4% PFA fixed lungs were placed in an amplifying hydrogel solution (AHS, 20% acrylamide, 0.05% bis-acrylamide, 4% paraformaldehyde, 10% sodium acrylate, 0.1% VA-044 in 1× PBS), and incubated on a horizontal shaker for 2–3 days at 4°C. Then, the samples were covered with AHS solution in a 6-well plate and incubated in a humidified chamber at 37°C for 2 hours until the solution turned into a gel. The tissue containing gels were cut into 500 μm sections with a vibratome, and then stored in 1× PBS at 4°C until further processing. Tissue sections were covered with freshly prepared denaturation solution (200 mM sodium dodecyl sulfate, 50 mM Tris [pH 9.0], 200 mM NaCL in 1× PBS) in a 6-well plate and incubated for 24 hours at 70°C, and then for 12 hours at 95°C in a humidified chamber. At last, the slices were washed with 1× PBS
for 2 hours (refresh PBS every 30 min) on a horizontal shaker.

Samples were incubated in blocking buffer (2% BSA and 1% Triton-X-100 in 1x PBS) for 1 day at 37°C. Then, they were covered with primary antibodies (Rabbit anti-AQP5, 1:100, Abcam, catalog # ab78486; Goat anti-CD31, 1:100, RD systems, catalog # AF3628; Rabbit anti-Acetyl-alpha-Tubulin [α-Ac-Tub], 1:100, Sigma-Aldrich, catalog # SAB5600134; Rat anti-Ly-6G/Ly-6C, 1:50, Invitrogen, catalog # MA1-10401) diluted in blocking buffer at 37°C for 2 days. The first antibody stained samples were washed in blocking buffer three times (1 hour for each time) at 37°C. After that, samples were incubated with the secondary antibodies (Donkey anti-Rabbit IgG-Alexa Fluor 568, 1:100, Abcam, catalog # ab175470; Donkey anti-Goat IgG-Alexa Fluor 647, 1:100, Abcam, catalog # ab150131; Donkey anti-Rat IgG-Alexa Fluor 647, 1:100, Abcam, catalog # ab150155) diluted in blocking buffer at 37°C for 2 days. Finally, the samples were washed with 1x PBS three times (1 hour for each time) at 37°C and stored in 1x PBS at 4°C until imaging (Figure 1E, ExM). Far-infrared fluorescence conjugated secondary antibodies are recommended to avoid the tissue autofluorescence interference.

**Deparaffinization of paraffin-embedded blocks**

Tissues were cut out with a blade from paraffin blocks and heated in an incubator at 65°C until paraffin was macroscopically removed. Then, the samples were washed with xylene for 12–24h at room temperature on a rotary. After deparaffinization, samples were rehydrated with 100%, 90%, 80%, 70% ethanol diluted with ddH2O for 1 hour respectively. Before being subjected to ExM, samples were washed with 1x PBS overnight.

**Microscopy and data management**

Samples were imaged using Leica SP8 LCFM or LSFM. The specimens were imaged with a 488-nm laser for the autofluorescence scanning; for the detection of MHI148-PEI, Alexa Fluor 568 and Alexa Fluor 647-conjugated antibodies, samples were excited with 638-nm, 552-nm and 638-nm lasers, respectively. ECi based OTC: ①LCFM: samples were mounted in a Petri dish with glue and immersed in immersion oil (RI ≈ 1.51, nearly the same to the RI of ECi). ②LSFM: samples were mounted on a parafilm made base in central part of a petri dish with glue. After immobilization, ECi was added carefully as RI matching medium. ExM: Gel slices were transferred to a petri dish and were immobilized with 1% agarose to prevent a drift during imaging. The whole slices were almost transparent and its RI matches to water (RI ≈ 1.33), so 1x PBS was used as immersion medium. Images were obtained with HC PL APO 20×/0.7 IMM CORR CS2 or HCX PL APO 63×/1.2 Water CS objective of the LCFM, or HC PL FLUOTAR 5×/0.15 IMM DLS objective of the LSFM.

**Data management**

All of the pictures and movies were created by the LAS X software (Leica microsystems, Mannheim, Germany). The tiles of z-stacks were stitched and rendered into 3D volume automatically by the LAS X software too. All the data acquired were stored in the Scientific Data Storage (SDS, University of Heidelberg) where the data can be accessed at high speed.

**Quantitative analysis**

1. The maximum alveolar diameter in different planes of 3D images were measured by LAS X software, and the average value and standard deviation were calculated as the average diameter of alveoli. The normal alveolar diameter were measured and calculated with several H&E stained lung slices in the same way.
2. For the quantitative analysis of neutrophil cells distribution in lung metastases, Fiji and MATLAB software were applied. Maximum intensity projection image after 3-class classification was achieved by Fiji plugin, Tainable Weka Segmentation. X×Y (40×40) was defined as boxes, and the total pixels of neutrophil cells in 3D were calculated for each point by MATLAB. Finally, the histogram of neutrophil cells distribution was smoothed by Gaussian filter with std deviation = 2.
Results

**3D Imaging of normal lung vasculature after ECi based OTC**

The lung showed a high degree of optical transparency after ECi based OTC (Figure 1C1). The volume change was estimated by calculating the diameter of alveoli. A decrease of alveolar diameter (about 30.6%) after ECi based OTC was observed (Figure 1D). As illustrated in Figure 2B, the MHI148-PEI labeled lung vasculature was clearly revealed using LCFM. By autofluorescence imaging, we can also get a rough 3D structure of the alveoli (Figure 2A). Figure 2C and 2D show the overlay and depth coding of vessels giving a better understanding of the vasculature.

We also imaged the MHI148-PEI perfused and ECi cleared lung by LSFM. Compared with the
results by LCFM, we got a more comprehensive structure of lung vasculature, but with a lower magnification. Figure 3A shows the structure of the lung obtained by autofluorescence scanning, in which the tube-like structure with high green fluorescence intensity in the central part indicates bronchi. We successfully visualized the vasculature in lung at a depth of >1.4 mm (Figure 3B and 3D). Figure 3C gives the overlay of autofluorescence and dye scanning in which we can detect the vasculature around bronchi in 3D.

In order to promote the application of ECi based OTC, we ever did 3D immunofluorescence staining before and after ECi clearing with lung. Even though the antibody staining works well in both of them, the alveoli were mostly deformed (Supplementary Figure 1). Therefore, we decided to establish another 3D imaging protocol for lung, by which the alveoli should be well preserved.

**3D Immunofluorescence visualization of the normal lung by ExM**

We tried to reveal the 3D lung structure by ExM using anti-AQP5, anti-CD31 and anti-α-Ac-Tub antibody immunofluorescence staining, which are used to detect the type I alveolar epithelial cell, vascular endothelial cell and the cilia of bronchial epithelium in conventional 2D slice staining. We found that those antibodies could also be used in 3D imaging. As we can see in Figure 1C2, 500 μm thick mouse lung tissue showed a high degree of transparency after denaturation. The ExM results in an expansion factor of approximately 1.8 calculated by the changes of

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**Figure 3.** 3D visualization of MHI148-PEI labeled vasculature in normal lung processed by ECi based OTC (LSFM, 5x objective). (A) 3D profile structure of lung tissue (green) detected by autofluorescence scanning. The white arrows indicate bronchi. (B) 3D reconstructed image of vasculature (red) in normal lung, as indicated by white arrows. (C) Overlay image of the autofluorescence and MHI148-PEI scanning. (D) Depth coded image of the fluorescence labeled lung vasculature (B).
alveolar diameter (Figure 1D). In Figure 4A, the green autofluorescence indicated the profile structure of lung that contains alveoli, bronchi and vessels. The 3D structure of alveoli (Figure 4B) and vascular branches (Figure 4C) in lung were visualized by anti-AQP5 and anti-CD31 antibody staining after ExM. The three channels overlay (Figure 4D) allowed visualization of the spatial association between bronchi, alveoli and vessels.

Furthermore, by a high magnification 63× objective, we could get a more clear 3D image of alveoli made up of type 1 alveolar epithelial cells and capillaries going through the interstitial tissue (Figure 5, see also Supplementary Movie 1). We also applied anti-α-Ac-Tub antibody, a marker of ciliated cells on bronchial epithelium, into our experiment. As shown in Figure 6, anti-α-Ac-Tub antibody stained bronchial cilia were uniformly distributed throughout the surface of bronchial epithelium (see also Supplementary Movie 2).

3D Imaging of the microvasculature in paraffin-embedded lung metastases

We used ExM as a tool for 3D imaging of paraffin-embedded lung metastases blocks. 3D
reconstruction of the intense tumor capillary bed could be achieved, which showed a precise and integral map of the microvascular network (Figure 7A). It is interesting that the metastasis nodule was revealed automatically with a clear circular boundary after ExM. Figure 7B is the high magnification image of the highlighted area (white box) in Figure 7A to show in detail the complex structure of vasculature in lung metastases. As shown in Figure 7C, the dark area in the central part of tissue indicates that the sample is out-of-focus plane; however, the normal lung around tumor nodule can be imaged in focus at this point. It meant that the surface of slice was not as flat as that was cut by vibratome before denaturation. We deduced that the lung metastases and normal lung tissue around were expanded unequally since they are made up of different component. At last, the metastases showed up automatically under confocal microscopy.

We further combined the anti-CD31 antibody with nuclear counterstaining with Sytox green. In Figure 8A, the lung metastasis nodules, which showed an accumulation of nuclear as compared with the normal lung tissue, can be easily distinguished. Figure 8B demonstrated that the vasculature in the metastasis nodules were definitely different from the normal lung tissue around (see also Supplementary Movie 3). The Sytox green and anti-CD31 antibody fluorescence staining were overlaid in Figure 8C gave us a better understanding of the metastases and vasculature distribution in lung metastasis.

**3D Visualization and quantitative analysis of the neutrophil distribution in paraffin-embedded lung metastases**

Immune cells, especially neutrophil cells, dynamically regulate tumor progression and remote...
organ metastasis. To further extend the applicability of the ExM protocol, we sought to investigate the efficacy of this method for detecting neutrophil cells invasion in lung metastases. Using ExM and anti-Ly6G antibody immunofluorescence staining, the 3D distribution of Ly6G-positive neutrophil cells in the lung metastases and adjacent lung tissue was successfully visualized (Figure 9). It is obvious that an increased number of neutrophil cells is observed in the lung metastases as compared with surrounding lung tissue, suggesting the active recruitment of neutrophil cells during lung metastasis development.

As shown in Figure 10A, the maximum intensity projection of Figure 9 was obtained after 3-class classification using Fiji plugin, Trainable Weka Segmentation. There is a distinct accumulation of neutrophil cells (purple) in the lung metastases, which is indicated by the area showing the lack of lung tissue (green). For 3D distribution plot, MATLAB was used. We were able to get a 3D plot of neutrophil cells distribution (Figure 10B) and a smoothed raw data using a Gaussian filter with standard distribution of 2 (Figure 10C), which obviously give a better understanding of the amount of neutrophil cells accumulation in lung metastases in 3D level. This analysis revealed a significant imbalance of neutrophil cells accumulation between the lung metastases and peripheral lung tissue.

Discussion
As the lung is a soft organ full of air and it is prone to collapse, special procedures must be considered in collecting and preserving the tissue, especially for the 3D morphological studies. There are many different methods of lung fixation/preservation: (1) right ventricular perfusion, (2) trachea instillation, (3) intrathoracic in situ fixation. However, none of these techniques
could completely meet the requirements of morphological study in lung research. The right ventricular perfusion can flush away blood completely, but severe edema may occur with inappropriate pressure. Lung collapse happens due to the loss of negative pleural pressure after heart exposure. Trachea instillation can keep the lung well inflated, but it always results in alveoli distortion by unstable pressure. Ochoa et al. reported that they extracted lungs after trans-cardiac perfusion combined with trachea instillation; however, the lungs were still unevenly expanded after collapsing during trans-cardiac perfusion.\textsuperscript{20} The intrathoracic in situ fixation can provide the best protective effect for alveoli since lungs are extracted after the intact thorax was fixed for nearly 1 week.\textsuperscript{21} However, it always takes such a long time to complete fixation, and blood is still in the lung.

In our retrograde perfusion protocol, we perfuse the mice via abdominal aorta to keep thoracic cavity intact, the most critical factor to maintain negative pleural pressure that ensures lung expanded, through the whole process.\textsuperscript{22} A compressor and a syringe pump provide a stable perfusion pressure. Furthermore, after perfusion, the cervical part of trachea was isolated and ligated with a suture to avoid lung to collapse before opening thoracic cavity to extract the lungs. Based on the images of perfused lungs in Figure 2, 3 and Supplementary Figure 2, we demonstrated that lung morphology is pretty well preserved by our retrograde perfusion protocol combined with trachea ligation. The retrograde perfusion protocol also allows collecting all the other organs, which is consistent with animal protection welfare principles (3 R). Additionally, dyes can be infused to enable the in-situ staining of all organs at one time.

Figure 7. 3 D image of the microvasculature (white arrows) in paraffin-embedded lung metastases after ExM (LCFM, 20× objective). (A) 3 D image of the complex capillary network (red). The metastasis nodule was revealed automatically with a clear circular boundary. (B) High magnification image of the rectangle indicated area in A shows in detail the complex vascular network in lung metastases. (C) The dark area in the central part indicated that lung metastasis nodule is out-of-focus, however, the normal lung around can be imaged in focus. It demonstrated that the lung metastases and normal lung tissue around were expanded differently. Autofluorescence (green) was used to identify lung tissue profile.
The intrinsic autofluorescence arises from endogenous fluorophores, such as mitochondria, lysosomes and collagen, which are unevenly distributed in tissue. Even though it is often considered as noise, some studies demonstrated that the autofluorescence could be applied in histology research without any external probes.23,24 In our experiments, we imaged the samples with a 488-nm laser to catch tissue autofluorescence, which was a powerful tool to distinguish the profile structure of lung.

Different from other organic solvent-based OTC techniques, ECi is a cheap and nontoxic solvent that is quite suitable for an efficient clearing. It is also becoming popular for clearing other organs, such as heart, bone, eye and organoids.25–28 However, there is not any specific report about the ECi based OTC in lung research. In the previous study, our group optimized the ECi based OTC protocol from one day or several weeks to only 4 hours without any compromised clearing efficacy.14 Here we demonstrate that the

Figure 8. 3D imaging of paraffin-embedded lung metastases tissue co-stained with Sytox green and anti-CD31 antibody after ExM (LCFM, 20× objective). (A) The lung metastasis nodules (white arrows) showed an accumulation of nuclear (blue) as compared with the normal lung tissue around. (B) 3D image of the complex vascular network (red) in lung metastasis nodules, which is as indicated by the white arrows and different from the normal lung tissue around. (C) Overlay image of the Sytox green and anti-CD31 antibody staining.
optimized ECi based OTC protocol also works well with lung tissue. Pulmonary vascular remodeling is reported in the progression of many chronic lung disorders, such as asthma, chronic obstructive pulmonary disease, pulmonary hypertension and lung transplantation. Therefore, this protocol could be a powerful tool for the research of vascular remodeling-related lung diseases, especially with regard to the whole mount structures with 3D projection.

We tried to combine the ECi based OTC with conventional antibody staining to extend the scope of its application. Even though the immunofluorescence staining works well before and after ECi clearing, the alveoli were mostly deformed (Supplementary Figure 1). The main reason is that the structural stability of alveoli was destroyed due to the long-term immersion in antibody staining and washing. However, it is unavoidable that it always takes quite a long time to complete 3D immunofluorescence labeling because of the low antibody penetration rate in thick tissue. We conclude that the ECi based OTC is a promising OTC method; however, its application in lung research is limited when we try to combine it with antibody staining. Then, we decided to establish another 3D imaging protocol for lung tissue. Zhao et al. ever applied a modified ExPath protocol on 4 μm slices of normal lung and lung cancer, but the quality of the 2D images were poor and the information obtained by these thin slices were limited. Based on the principle of embedding tissue in a dense polymer network, we speculated that ExM protocol might be helpful to preserve alveolar morphology during the immunofluorescence labeling.

The majority constituents of lung are vascular endothelial cells and type I alveolar epithelial cells, so specific markers for them were chosen to perform 3D imaging of the lung. Although autofluorescence is always quite weak after ExM, it can still be used to detect the morphology of lung. Both of the large vessels and bronchi showed high intensity of autofluorescence in 3D, but we can recognize them individually with

Figure 9. 3D mapping of the Ly6G-positive neutrophil cells (red) distribution in paraffin-embedded lung metastases after ExM, as indicated by the white arrows (LCFM, 20× objective). Green: tissue profile structure identified by autofluorescence.
anti-CD31 antibody staining. 3 D high-resolution of alveoli and capillaries in the alveolar interstitium was visualized with ExM and immunofluorescence labeling with anti-AQP5 and anti-CD31 antibody, which has never been reported in previous research. However, only half of vessels in the lung tissue appears to be stained as shown in the Supplementary Movie 1, and the reason may be incomplete antibody penetration in the sample. A more robust labeling method is urgent to broaden the application of 3 D imaging, which should be addressed in the future. We hypothesize that the pulmonary microvascular and alveolar epithelial permeability may be analyzed by 3 D imaging with specific marker labeling directly instead of indirect measurement methods and indicators, such as dry/wet ratios, Evans blue fluorimetry and albumin concentration ratios between blood and bronchoalveolar lavage fluid.\textsuperscript{32,33} Considering the 3 D image of cilia distribution on bronchia, our protocol is also recommended to reveal the ciliary abnormalities caused by congenital or exogenous factors, such as cystic fibrosis, virus, bacteria and cigarette,\textsuperscript{34,35} and it can definitely give a comprehensive overview.

Since the tissue size uniformly expands nearly two times after denaturation, the resolution of images in our experiment is almost 2x2x2 times higher than in the traditional histological methods under same objective. A higher expansion rate can be achieved by swelling samples with ddH\textsubscript{2}O;\textsuperscript{36} however, the density of proteins turns to be too sparse upon excessive expansion in

**Figure 10.** 3 D quantitative analysis of neutrophil cell distribution in the lung metastases and adjacent lung tissue by Fiji and MATLAB. (A) Maximum intensity projection of Fig.9. Green: lung tissue; red: background; purple: neutrophil cells. (B) 3 D plot of neutrophil cell distribution using 40x40 pixels grids: Raw data. (C) The smoothed raw data of neutrophil cell distribution. The color bar represents the total pixels occupied by neutrophil cells.
Therefore, we recommend to limit the ExM to two fold, and to analyze samples after denaturation combined with a high magnification objective. In order to ensure that the samples could be imaged by LCFM, which is always equipped with short working distance objectives, after ExM, the specimens were sliced into 500 µm sections. Therefore, it may be not able to do 3D high-resolution imaging of an intact lung by ExM until a long enough working distance objective is developed.

In order to make a wider use of the ExM protocol, 10 years old paraffin-embedded lung metastases blocks were dewaxed and rehydrated, and then processed with ExM. The tumor vasculature is disorganized, malformed, tortuous, and randomly interconnected as demonstrated in our 3D data. We also found that the metastasis nodules were revealed automatically with a clear boundary after ExM, which has never been reported in other 3D imaging methods with respect to lung metastases. The reason may be that the lung metastases and normal surrounding lung tissue were expanded differently since they are made up of different kinds and amount of cells and matrix. The excess cells and matrix of the metastases absorbed more water as compared with the surrounding tissue, which resulted in a dome-like shape. This may be helpful to detect micro-metastases in lung tissue.

Immune cell content and distribution in the tumor microenvironment can provide valuable information about metastasis, prognostic and possibly therapeutic strategy in cancer. Our ExM protocol enables to reveal the 3D high-resolution spatial distribution of neutrophil cells in lung metastases. By quantitative analysis using Fiji and MATLAB, we were able to quantify the cell density and verify the distinct localization of neutrophils in lung metastases and adjacent lung tissue on a 3D level. Since the neutrophils are tightly packed into clusters, we could not obtain the actual number of neutrophil cells in the tissue. However, the total pixels of neutrophil cells by our statistical analysis method can definitely reflect the actual amount of neutrophils. Lung metastases biology and microenvironment can vary between the surface and central part, especially compared with the tumor adjacent and healthy tissue. Therefore, we highly recommend applying our ExM protocol to enable a comprehensive analysis of lung metastases in the future.

Analysis of selected tissue sections in clinical pathologic diagnosis is prone to unavoidable biases since important biomedical information may locate elsewhere. In contrast, 3D imaging of intact specimens after OTC or ExM could provide a much comprehensive information and greater level of insight into anatomy and pathology. For example, 3D labeling and imaging of human biopsy tissues from patients suffered from cancer or nephritis may provide a more accurate diagnosis and staging. Furthermore, the 3D imaging methods may also improve the antitumor drugs development by providing unbiased readouts of drug distribution in entire organs of treated and control groups.

In conclusion, the retrograde perfusion technique combined with trachea ligation, which results in a better maintenance of lung morphology, can be widely used in pulmonary pathology research in mice. 3D distribution of lung vasculature can be imaged by MHI148-PEI perfusion and ECI based OTC in a time saving and highly efficient way. ExM and immunofluorescence staining protocols, which work well with conventional antibodies and are helpful to preserve the alveolar morphology, are highly recommended for the 3D imaging of freshly fixed lung tissue and even paraffin-embedded lung tissue.

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Declaration of interest
The authors report no conflicts of interest.

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**ORCID**
Quanchao Sun http://orcid.org/0000-0003-0165-4834

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