

Visualization of Lung Vessels in Optically Cleared Specimens Using Second Harmonic Generation

S.A. Portnov^{1,A}, S.S. Shalyapin^{2,A}, A.O. Bogorodskiy^{3,A}, V.I. Borshchevskiy^{4,B},
M.A. Shevchenko^{5,C}

^A National Research Nuclear University MEPhI (Moscow Engineering Physics Institute)

^B Research Center for Molecular Mechanisms of Aging and Age-Related Diseases, Moscow
Institute of Physics and Technology

^C Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry RAS

¹ ORCID: 0009-0000-7978-9486, portnov.hb@gmail.com

² ORCID: 0009-0005-6101-3133, shalyapin956@gmail.com

³ ORCID: 0000-0002-7589-7823, bogorodskiy173@gmail.com

⁴ ORCID: 0000-0003-4398-9712, borshchevskiy.vi@phystech.edu

⁵ ORCID: 0000-0001-5278-9937, mshevch@gmail.com

Abstract

Second harmonic generation (SHG) is a nonlinear optical process, and SHG microscopy is a key method for imaging in biomedical science. In the present study, we evaluated the usage of SHG for the imaging of the airways and vessels in the optically cleared mouse lung. SHG visualized F-actin and mainly collagen fibers bordering both airways and vessels. For distinguishing airways from vessels, we used additional staining of the airways with Alexa[®]Fluor[®]488-labeled streptavidin and developed the image processing technique for the separation of Alexa[®]Fluor[®]488 and SHG signals.

The approach described here allows a high-resolution imaging of anatomic structures of the lung of small animals and can be further used for drug and pathogen detection in experimental disease models.

Keywords: lung vessels, mouse model, tissue optical clearing, second harmonic generation, two-photon microscopy.

1. Introduction

Daily, people inhale pathogenic and anthropogenic particles and exogenous molecules that disseminate through the respiratory tract; some of them can penetrate the blood vessels and invade the whole organism. Depending on their location, pathogens can induce local or systemic inflammation [1, 2].

Detection of the precise location of pathogens or drugs in the lungs during an inflammation is essential for the development of the optimal treatment approach. Different experimental models are explored for this purpose. Mouse models of the diseases are well established and informative in the study of the inflammatory properties in the lungs. Moreover, imaging of the whole-mount organs, e.g., lungs, of the small laboratory animals such as mice is a convenient and perspective technique [3].

Confocal laser scanning microscopy is a perfect tool for the visualization of tissues and organs of laboratory animals. However, the limitation of the approach is the laser light-scatter; therefore, imaging of deep tissue regions is limited to 100 μm . Using two-photon microscopy with long wavelengths (800–1200 nm) reduces sample damage and allows for imaging deep tissue regions (250–500 μm) [4, 5].

Tissue clearance can help to avoid the loss of resolution. Multiple tissue clearing techniques were developed during the last century. Mostly, these methods are based on the treatment of specimens with organic solvents or hydrogels [4, 6].

To properly visualize pathogens or encapsulated drugs in the depth of tissues or organs, researchers use fluorescent labels that must be bright and stable. Moreover, immune cells and structural tissue cells should also be visualized. Confocal and two-photon microscopy are characterized by autofluorescence that can be used for the detection of metabolic molecules such as NADH and tryptophan. On the other hand, autofluorescence can give a false-positive signal and limit the number of potential fluorophores for cell, pathogen, and drug detection [5, 7].

Second harmonic generation (SHG) microscopy, which is based on two-photon microscopy, has become a key label-free technique for imaging with many applications in biomedical sciences. SHG microscopy is a non-fluorescent technique. SHG has been reported for a variety of molecules (SHG active molecules a.k.a. harmonophores), such as collagen, myosin, microtubules, silk, starch, and cellulose. These molecules have non-centrosymmetric structures and can form filaments. In the biological specimens, SHG can be used in combination with fluorescent staining [5, 8].

In the present study, based on the combination of SHG and fluorescent two-photon microscopy, we developed an approach for imaging the airways, vessels, and pathogens in the optically cleared mouse lungs.

2. The approach to visualization of the airways, vessels, and pathogens in the optically cleared mouse lung

2.1. Mouse lung specimen preparation

To visualize pathogens in the lung, particularly inside the airways and vessels, spores of opportunistic fungi *Aspergillus fumigatus* were in advance labeled with succinimidyl ester of Alexa Fluor 594 as we described previously [9]. Spores were then applied to mice via the oropharyngeal route in a total volume of 50 μL per mouse and a dose of 1×10^8 spores/mL. Twenty-four hours after, mice were sacrificed with cervical dislocation and the lungs were extracted, fixed with 2% paraformaldehyde overnight, stained with Alexa[®]Fluor[®]488-conjugated streptavidin, and underwent optical clearing using a 1:2 v/v mixture of benzyl alcohol and benzyl benzoate. Mouse lung lobe before and after optical clearing is presented in the Figure 1 (a) and (b), respectively.

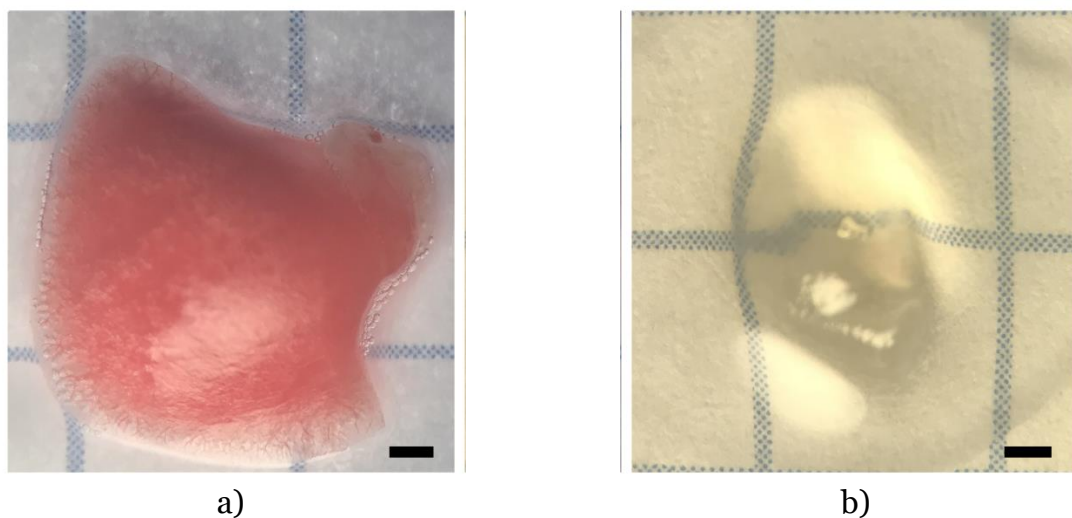


Fig. 1. Right superior lobe of the mouse lungs before (a) and after (b) optical clearing.

Within 24 h optically cleared lung lobe underwent examination using two-photon microscopy.

2.2. Image acquisition using two-photon microscopy

In the present study, we used an inverted Zeiss LSM 780 that was additionally equipped with an Insight DeepSee-pulsed solid-state laser with ultrashort pulses (<150 fs) and a spectral range from 680 nm to 1300 nm, which enables two-photon microscopy. The laser system can be used for different purposes, such as fluorescence lifetime measurements, cell and tissue imaging [10-12].

For the simultaneous visualization of Alexa[®]Fluor[®]488 and Alexa[®]Fluor[®]594, the optimal wavelength for the two-photon laser was selected. In two-photon microscopy, each photon has half the energy of the corresponding single-photon absorption event [13]. In accordance with this (see formula (1) and Figure 2), the optimal wavelength for the effective excitation of Alexa[®]Fluor[®]488 is 976 nm, and for Alexa[®]Fluor[®]594 - 1188 nm. To visualize Alexa[®]Fluor[®]488 and Alexa[®]Fluor[®]594 simultaneously, the optimal wavelength was selected in the range from 976 to 1188 nm empirically and set to 1050 nm.

$$\lambda_{2P} = 2 \times \lambda_{1P} \quad (1),$$

where λ_{1P} – is the wavelength of single-photon excitation,
 λ_{2P} – is the optimal wavelength for two-photon excitation.

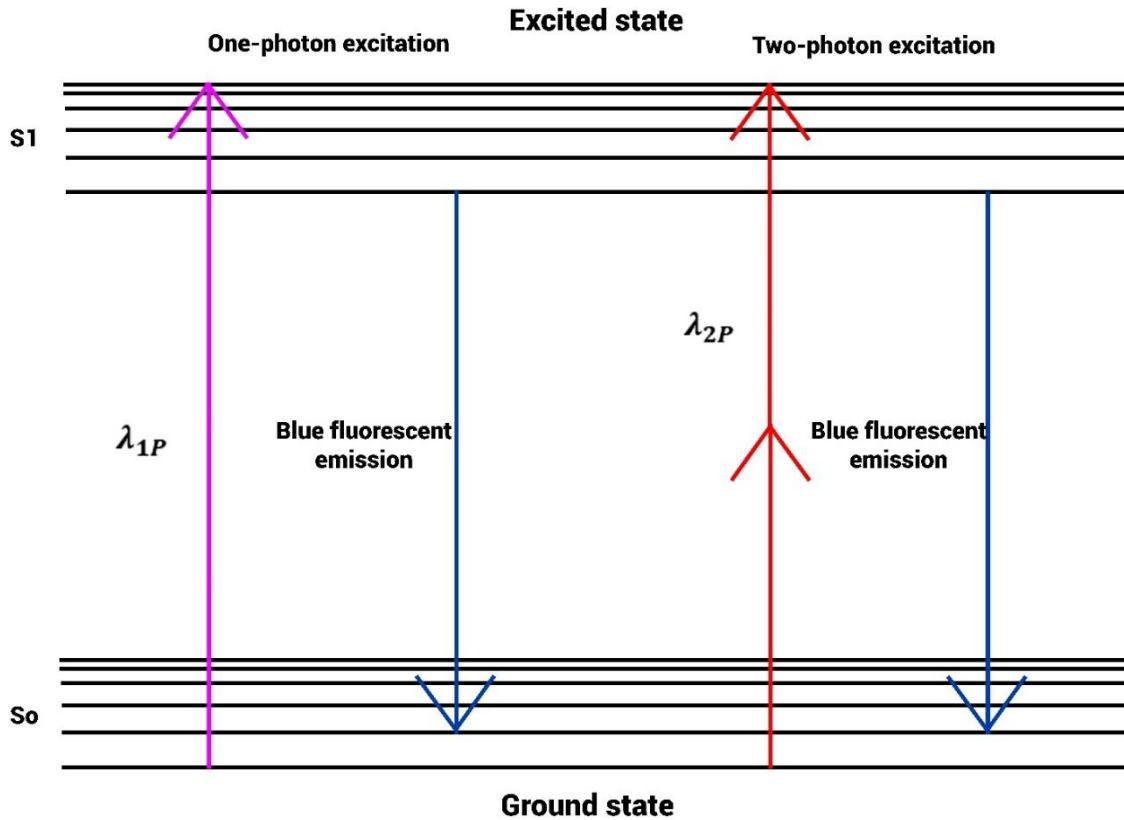


Fig. 2. The Yablonsky diagram. One-photon excitation (left) and two-photon excitation (right) (adopted from [13]).

Zeiss LSM 780 is equipped with a 34-channel QUASAR detector (Zeiss), ranging from 490 to 695 nm. Based on the analysis of the emission and excitation spectra of Alexa[®]Fluor[®]488 and Alexa[®]Fluor[®]594, we used only 9 channels (490, 508, 526, 544, 561, 579, 597, 615, and 633 nm) that enabled an essential reduction of the file size and processing time.

To obtain high-resolution images, $\times 10$ (NA =0.3) or $\times 20$ (NA =0.5) objectives were used. Images were acquired in Tile-Scan mode as Z-stacks, with a resolution of 512×512 and a Z-step of $5 \mu\text{m}$.

Before further processing, images underwent spectral unmixing, which permitted the achievement of the SHG spectra. The emission peaks of Alexa[®]Fluor[®]488 and Alexa[®]Fluor[®]594 were observed at approximately 530 and 610 nm, respectively, as expected from the known data of the fluorophore emissions. The SHG emission spectrum was obtained at 515 nm. Emission spectra are shown in Figure 3.

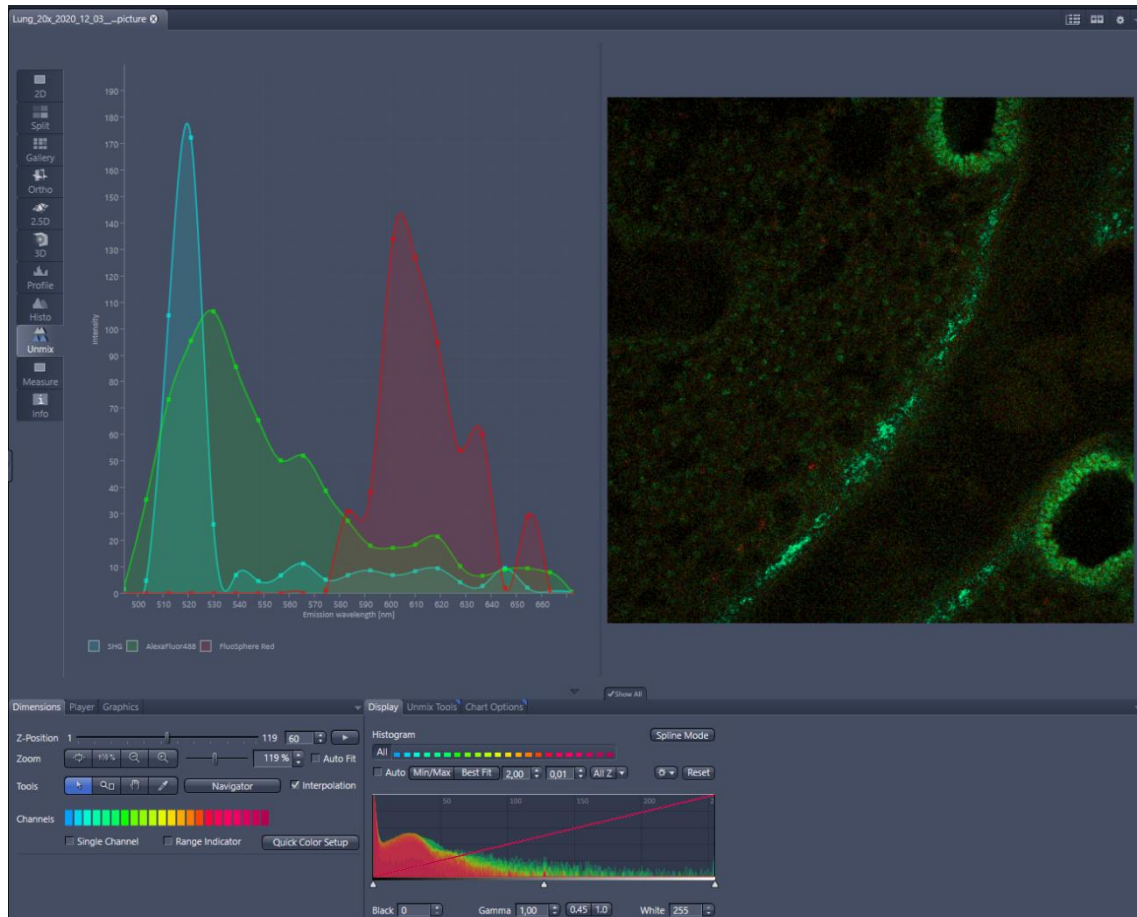


Fig. 3. Representative screen for spectral unmixing of SHG (cyan), Alexa[®]Fluor[®]488 (green), and Alexa[®]Fluor[®]594 (red).

2.3. Image processing using intensity mean thresholds

Three-dimensional images of the lung specimens were processed using Imaris 9.8 (Oxford Instruments, UK) software [14]. Imaris is the image analysis software, perfectly fitted for processing a large scale of three-dimensional images obtained with confocal laser scanning microscopy [15].

The following steps were performed in Imaris software:

- creation of a surface based on the Alexa[®]Fluor[®]488 channel;
- creation of a surface based on the SHG channel;
- dividing the SHG surface into segments with an approximate diameter of $20 \mu\text{m}$;
- filtering the segments using the “Intensity Mean” threshold in the channel of Alexa[®]Fluor[®]488;
- creation of a mask based on the obtained surface (the surface is referred to hereafter as the vessel surface);
- creation of spots based on the Alexa[®]Fluor[®]594 channel;

- filtering the spots using the “Intensity Mean” threshold in the channel of the vessel surface mask.

The surfaces were created using the “Create Surface” option as we described previously [9]. This stage also allowed to remove the noise from the image obtained after spectral unmixing.

When dividing the SHG surface into segments, the option “Seed Points Diameter” was used. The optimal approximate diameter of the segment was set to 20 μm based on the relation between the time of the processing and the accuracy of the vessel surface.

Filtering was performed using an “Intensity Mean” threshold that split all segments based on the emission intensity into associated and not associated with the Alexa[®]Fluor[®]488 channel. These were not associated were selected by us as corresponding to the vessels. Based on the selection vessel surface was created.

The same principle was applied when detecting pathogen (*A. fumigatus* spores) inside the vessels. Spots of the pathogen were split into associated and not associated with the vessel.

3. Visualization of vessels using SHG in a whole-mount lung specimen

3.1 SHG illuminated branched structures of airways and vessels in the three-dimensional lung lobe specimen images

Spectral unmixing was performed as described in Chapter 2.2. Images in SHG and Alexa[®]Fluor[®]488 channels for the whole-mount mouse right lung lobe specimen are presented in Figure 4.

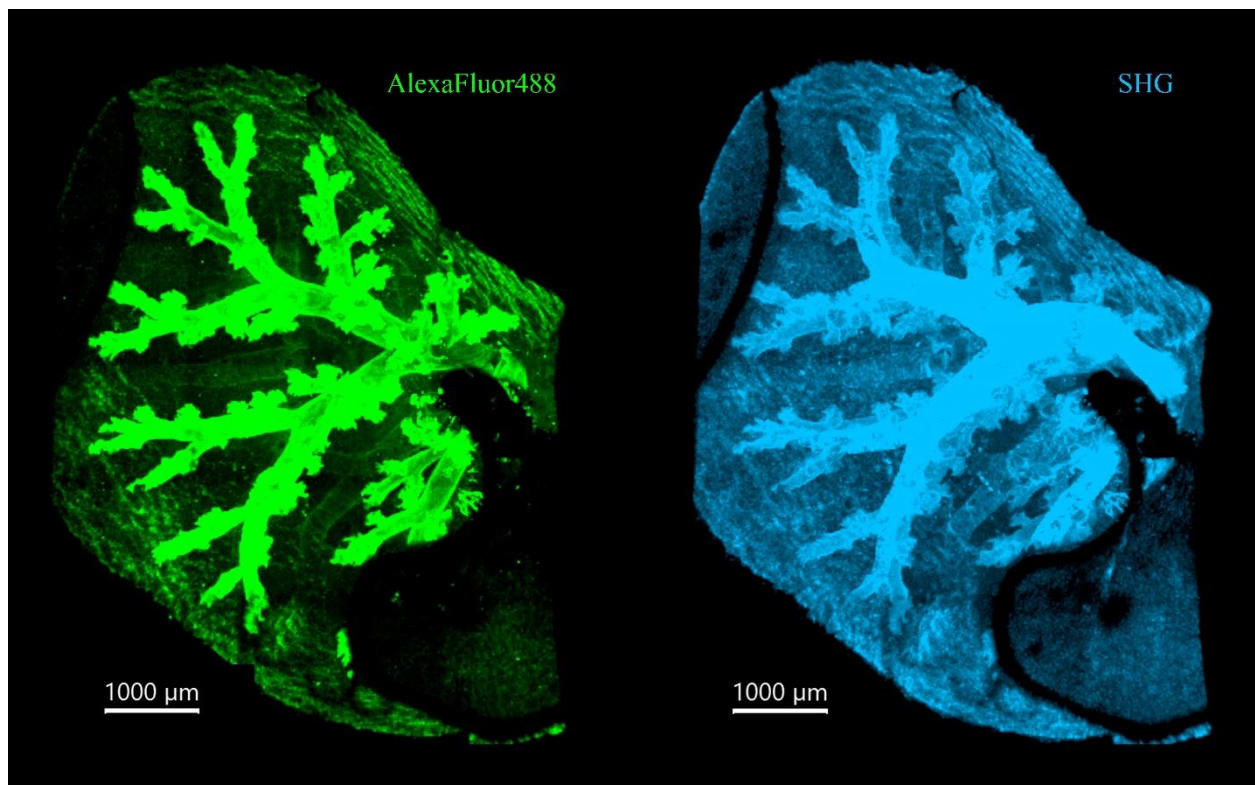


Fig. 4. Representative images of Alexa[®]Fluor[®]488 (green, left) and SHG (cyan, right) channels obtained after spectral unmixing. Scale bar 1000 μm .

It has been observed that the SHG channel illuminated an additional structure compared to the image in the Alexa[®]Fluor[®]488 channel. Indeed, the SHG channel was expected to include both the respiratory tract and blood vessels due to the airway wall and endothelial col-

lagen anisotropic properties triggering SHG. On the other hand, Alexa°Fluor°488 allowed visualization of the respiratory tract only (due to the streptavidin-biotin binding).

3.2. Segment classification allowed obtaining the lung vessel surface based on the image in the SHG channel

Based on the SHG channel obtained using the spectral unmixing, the SHG surface was created and divided into segments with an approximate diameter of 20 μm , as indicated in Figure 5 (a).

The Intensity Mean filter in the Alexa°Fluor°488 channel was applied to split the segments of the SHG surface: segments corresponding to the Alexa°Fluor°488 surface were stained with cyan, and the rest with magenta, as indicated in Figure 5 (b).

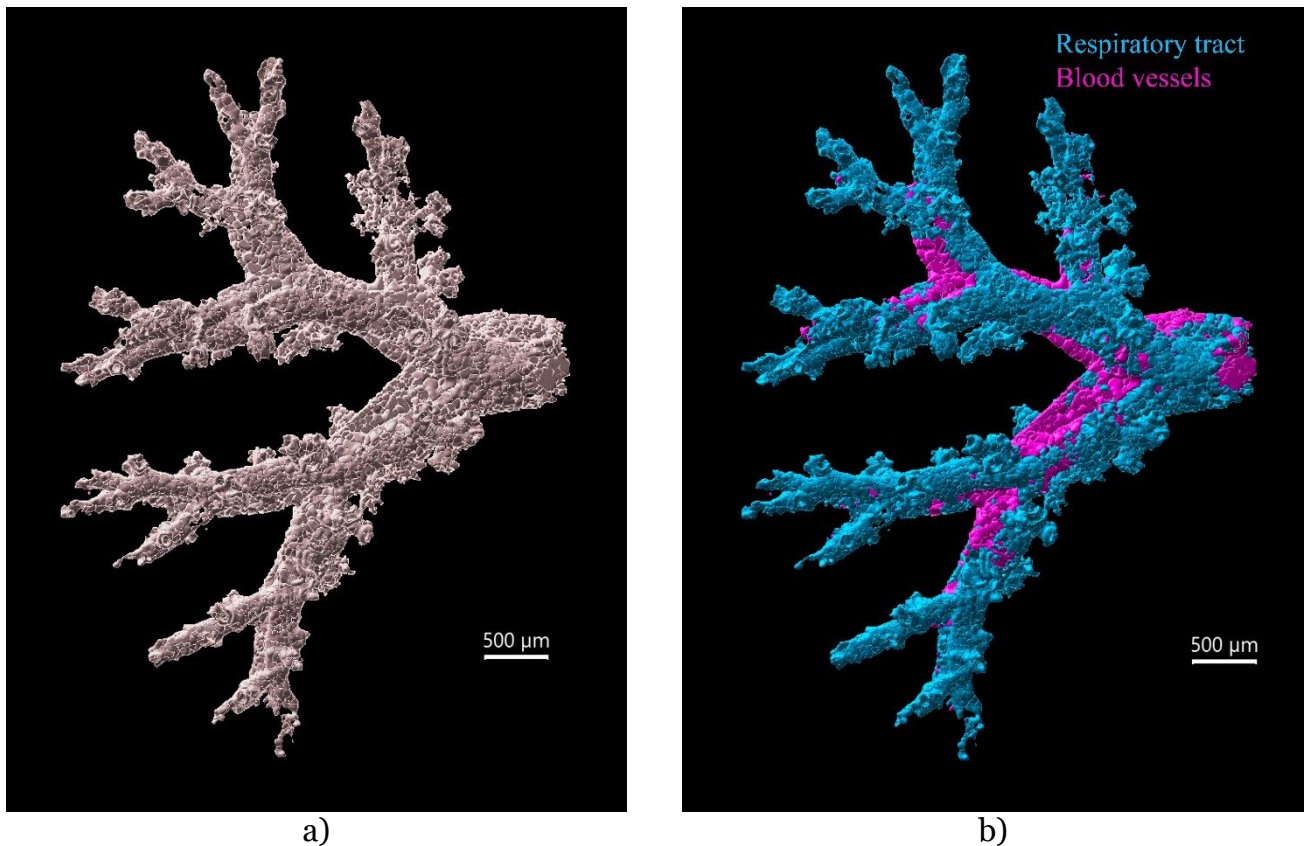


Fig. 5. SHG surface was divided into the segments (a), and the segments were classified as associated with Alexa°Fluor°488 channel (cyan) and not associated (magenta) (b). Scale bar 500 μm .

To obtain the surface of the vessels, we manually removed the segments belonging to the airways (indicated with cyan in Figure 5 (b)) using the “Delete” option in the graphic section. The segments indicated in magenta were left; they form the surface of lung vessels. To ensure accuracy, the process of segment removal was verified against the Alexa°Fluor°488 surface to avoid removing unnecessary segments.

4. Detection of pathogens in the lung vessels

The creation of the blood vessel surface was necessary for the accurate identification of *A.°fumigatus* fungal spores in the lung, and particularly in blood vessels.

To do this, the mask was created based on the vessel surface using the “Mask Selection” option. Spores were created using the “Spots” option, spot diameter was selected as 5 μm , and PZF 10 μm .

Filtering of the obtained spots did not reveal spots inside the vessels, as shown in Figure 6.

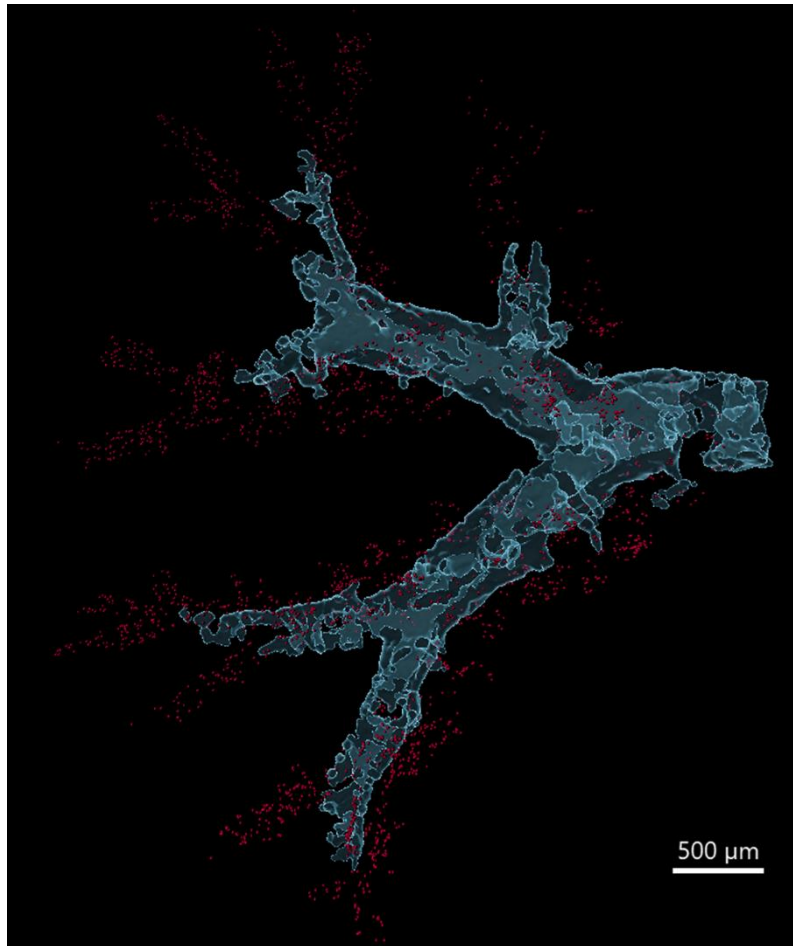


Fig. 6. Representative image of the blood vessel surface (transparent blue) and *A. fumigatus* spores (red). Scale bar 500 μm .

Thus, our data indicated that *A. fumigatus* spores could not penetrate blood vessels in an immunocompetent organism (mouse).

5. Conclusions

Here, we proposed an approach that allows a high-resolution imaging of the airways and vessels in the optically cleared lung of experimental mice using two-photon microscopy and SHG. Subsequent image processing was also developed in the present study.

The approach combined several experimental techniques: biological specimen preparation, specimen imaging using two-photon microscopy, and image processing. For accurate specimen preparation and imaging, an initial literature analysis was performed. The simple and reliable techniques for immunohistochemistry and tissue optical clearing were used in the present study. Thus, airways were stained with fluorophore-conjugated streptavidin; the staining was based on the club cell capability of high biotin expression. A label-free technique, such as SHG, was applied to illuminate actin-rich and collagen-rich structures, including smooth muscles around the airways and endothelium. Although antibody staining was avoided in the present study, it can be used in combination with the proposed approach to visualize additional structures or immune cells in the lung vessels. As a result, using only one label, we visualized two structures; however, additional image processing was required to split these two structures.

The image processing technique was entirely developed in the present study and allowed for obtaining a separate structure of the airway-associated lung vessels. Subsequent analysis of in-advance labeled pathogens (spores of *A. fumigatus* fungi) revealed the absence of the pathogens in the lung vessels in the mouse model that was used in the present study.

The approach that was developed in the present study was successfully applied for investigating the ability of potentially pathogenic fungi to undergo systemic dissemination via the blood vessels. Using the approach, one can trace fluorescent drugs or specifically labelled immune cells in the vascular system of the lungs, brain, and spleen in different experimental animal models.

Acknowledgments

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