

Video Article

Direct Observation of Phagocytosis and NET-formation by Neutrophils in Infected Lungs using 2-photon Microscopy

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Abstract

After the gastrointestinal tract, the lung is the second largest surface for interaction between the vertebrate body and the environment. Here, an effective gas exchange must be maintained, while at the same time avoiding infection by the multiple pathogens that are inhaled during normal breathing. To achieve this, a superb set of defense strategies combining humoral and cellular immune mechanisms exists. One of the most effective measures for acute defense of the lung is the recruitment of neutrophils, which either phagocytose the inhaled pathogens or kill them by releasing cytotoxic chemicals. A recent addition to the arsenal of neutrophils is their explosive release of extracellular DNA-NETs by which bacteria or fungi can be caught or inactivated even after the NET releasing cells have died. We present here a method that allows one to directly observe neutrophils, migrating within a recently infected lung, phagocytosing fungal pathogens as well as visualize the extensive NETs that they have produced throughout the infected tissue. The method describes the preparation of thick viable lung slices 7 hours after intratracheal infection of mice with conidia of the mold *Aspergillus fumigatus* and their examination by multicolor time-lapse 2-photon microscopy. This approach allows one to directly investigate antifungal defense in native lung tissue and thus opens a new avenue for the detailed investigation of pulmonary immunity.

Video Link

The video component of this article can be found at <http://www.jove.com/video/2659/>

Protocol

1. Infection

1. Swollen conidia of the mold *Aspergillus fumigatus* (strain ATCC 46645) are generated by pre-incubation in RPMI 1640 (Biochrom AG, Berlin, Germany) supplemented with 5% FCS (v/v). 2×10^7 resting conidia are resuspended in 5 ml medium and incubated in a 6-well tissue culture plate (TPP AG; Trasadingen, Switzerland, catalog # 92006) for 7h at 37°C.
2. Following the swelling period, the spores are fluorescently labeled by adding 10 μ l of calcofluor white stock solution (Sigma Aldrich, Deisenhofen, Germany, catalog # F3543; [25 mg/ml] in DMSO) to each *Aspergillus* containing well in the 6-well plate, reaching a final calcofluor concentration of 50 μ g/ml RPMI. Following gentle mixing, the spores are incubated for an additional 15 min at 37°C.
3. In the next step, the spores are harvested by filtration through a 70 μ m cell strainer (BD Biosciences, Heidelberg, Germany). After washing once with 10 ml PBS (centrifuge at 900xg for 5 min at room temperature) the pellet is resuspended in 2 ml PBS and the total number of swollen conidia determined by counting with a Neubauer chamber. Finally the spore suspension is spun down at 900xg for 5 min at room temperature, the supernatant is carefully removed, and the pellet resuspended to the final concentration of 1×10^7 spores per 100 μ l PBS.
4. For infection, 100 μ l of spore solution is applied intratracheally into female mice (C57/BL6, 8–10 weeks old, Harlan, Germany). If neutrophils are to be observed as well, the use of Lys-EGFP mice, carrying EGFP under the lysozyme promoter¹ is recommended. Begin by anesthetizing the animal with a single i.p. injection of 150 μ l Ketamin/Rompun solution (Inresa Arzneimittel GmbH, Freiburg, Germany (Ketamin) and Bayer Vital GmbH, Leverkusen, Germany (Rompun), 1 ml Ketamin [50 mg/ml] + 0.5 ml Rompun [2 %] + 3.5 ml sterile NaCl [0.9 %]). 5 minutes later the anesthetized mouse can be fixed with an elastic band by its teeth to the ramp (i.e. a sloped surface) to facilitate the intubation (supplemental figure 1). Using forceps, the tongue is pulled to one side and a 22G indwelling venous catheter (B. Braun AG, Melsungen, Germany, Vasofix Braunüle) can be gently inserted into the trachea under permanent illumination with a goose neck lamp. The successful insertion of the catheter is verified by observing regular movement of the animal's thorax with the frequency of the mechanical ventilator. When the successful intubation has been confirmed, 100 μ l spore suspension is applied using a 100 μ l micropipette. This volume should be inhaled by the animal without any additional help in 1-2 s. An enhanced distribution of fungal particles inside the lung is achieved by mechanically ventilating the infected animal for 2 minutes with a small animal respirator (MiniVent, Hugo Sachs, March-Hugstetten, Germany) at rate of 250 breaths per minute and an inhalation volume of 300 μ l per breath (supplemental figure 2). After the infection animals

are returned to their cage and observed every 5 minutes until ambulatory again. Mice do not show any evidence of pain or distress during the following incubation period.

2. Lung preparation

1. 7 h after infection with the spores, the animal is euthanized by an overdose of isoflurane (Baxter GmbH, Unterschleißheim, Germany) until movements and breathing have ceased for more than 30 seconds. This is then followed by thoracotomy as a secondary method to assure clinical death and allow for visualization and access of the trachea and lungs. Then the chest is carefully opened to expose the lung and the upper respiratory tract. Then another 22G indwelling venous catheter is inserted into the trachea starting from the exposed epiglottis. Through this tubule the lungs are filled with 1 ml pre-warmed low melting agarose (2 % w/v, Promega, Mannheim, Germany) using a 1 ml Omnifix syringe (B.Braun). As soon as the lungs are filled the trachea is tied off with a short piece of sewing thread and the whole animal is put into a refrigerator at 4 °C.
2. 15 min later, when the agarose has solidified, the whole lung is excised beginning from the trachea. Using a pair of sharp pointed scissors, the right lung lobe is subsequently removed, the bottom surface is briefly dried on a sheet of tissue paper and then the whole lobe is glued to the preparation block of a vibratome (Campden Instruments, UK, 752M Vibroslice) using a drop of tissue adhesive for fixation (Carl Roth GmbH, Karlsruhe, Germany, Roti-Coll 1). After 1 min the block is installed in the cutting chamber of the vibratome, which is filled with pre-chilled (4°C) PBS. The vibratome is set to a mid-range vibration (5 out of 10 max.) and also moderate speed (5 out of 10 max.) such that a horizontal cross section of the lung is produced. The upper half of the organ is then transferred into a small plastic Petri dish (5 cm diameter) where it is inverted and fixed with a self-made flat washer (1 cm inner diameter) that is covered by a set of parallel nylon threads, each 1 mm apart (supplemental figure 3). Finally the Petri dish is filled with 10 ml PBS supplemented with 10 µl of the DNA dye Sytox Orange [5 mM] (Invitrogen, Germany) resulting in a final dye concentration of 5 µM.

3. 2-photon laser microscopy

1. The Petri dish containing the lung sample is then installed under the microscope objective so that the buffer can be warmed to 37°C by a temperature sensor-controlled heater (supplemental figure 4). 2-photon microscopy is then carried out over the whole cut surface using a Zeiss LSM 710 NLO microscope on an upright Axio Examiner stage equipped with a 20xNA1.0 water dipping lens (Zeiss, Jena, Germany). For imaging, different areas along the section are scanned down to 400 µm depth using an illumination wavelength of 800 nm detecting green (530 nm) and red (580 nm) fluorescence, as well as the second harmonic generation (SHG)-signal and the blue calcofluor fluorescence (at 400–470 nm emission) with external non descanned detectors (NDD). Besides 2-D images and time lapse movies (1 image every 5-10 seconds), single or repetitive 3-dimensional picture stacks can be subsequently rendered as voxel renderings or single extended focus images or 4-D data (3D over time) using different software packages. This microscopic setup allows the observation of cellular behavior in an almost intact environment that, as it constitutes the entry port for a variety of airborne pathogens, is of enormous immunological relevance. Cell motility, phagocytosis and NET production by endogenous neutrophils following infection with the mold *Aspergillus fumigatus* can be easily investigated under these in situ conditions.

4. Representative Results

If done properly the imaging will generate 2- or 3-color slides or movies. Although coloring is done in a post processing procedure and is thus freely adaptable, we generally select a coloring scheme that reflects the natural color of the dye/signal, which is detected in the relative channel. Thus, the Sytox dyes are depicted in red, the fungus as well as the SHG signal is shown in blue and, if present, EGFP-labeled cells are stained in green. In the first example (Fig. 1) the blue SHG signal depicts the tissue fibers of a non-infected lung and the red Sytox signal is produced by nuclei of lung-resident cells cut open by the vibratome. In the second example (Fig. 2) an infected lung is shown, where both, alveolar structures as well as fungal masses, appear in blue, while nuclei and NETs are stained red. The third example (Fig. 3) is from a Lys-EGFP transgenic animal where in addition to the blue and red structures the green neutrophils can also be seen. The migration of neutrophils and their phagocytosis of individual fungal elements in time lapse sequences is shown in the supplemental movie.

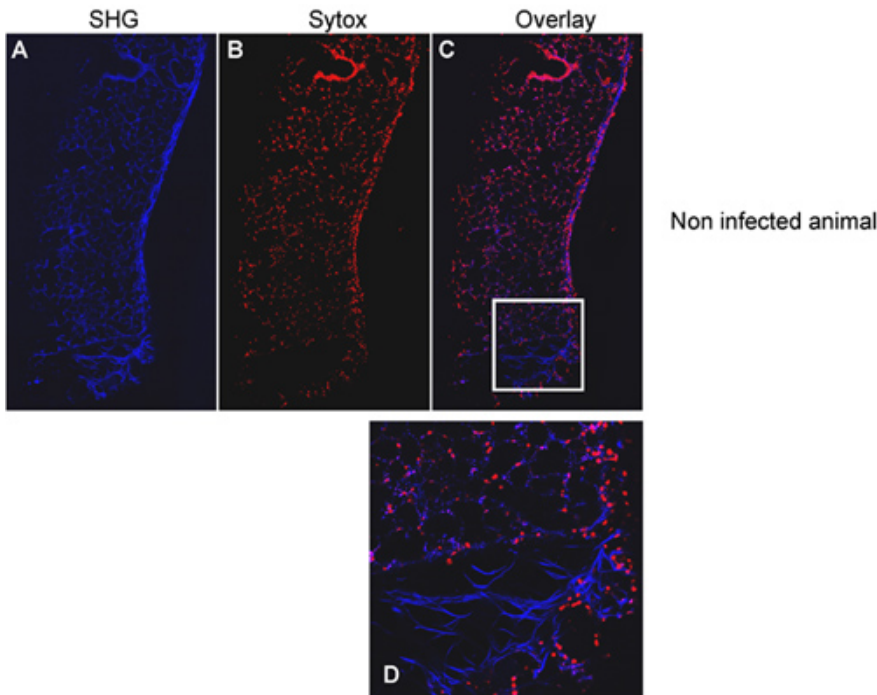


Figure 1. The appearance of a non-infected lung slice in 2-color 2-photon microscopy. A lung slice was prepared from a non-infected C57/BL6 mouse and imaged as described in the protocol. Presented here are the SHG signal of the alveolar tissue structure (A), the Sytox signal of cell nuclei cut open during the preparation of the lung slice (B), and an overlay of the two channels (C). The boxed area in (C) is seen enlarged in (D). Please note the fibrous tissue at the bottom of the lung slice as compared to the clearly alveolar organization within the breathing-active areas of the lung above.

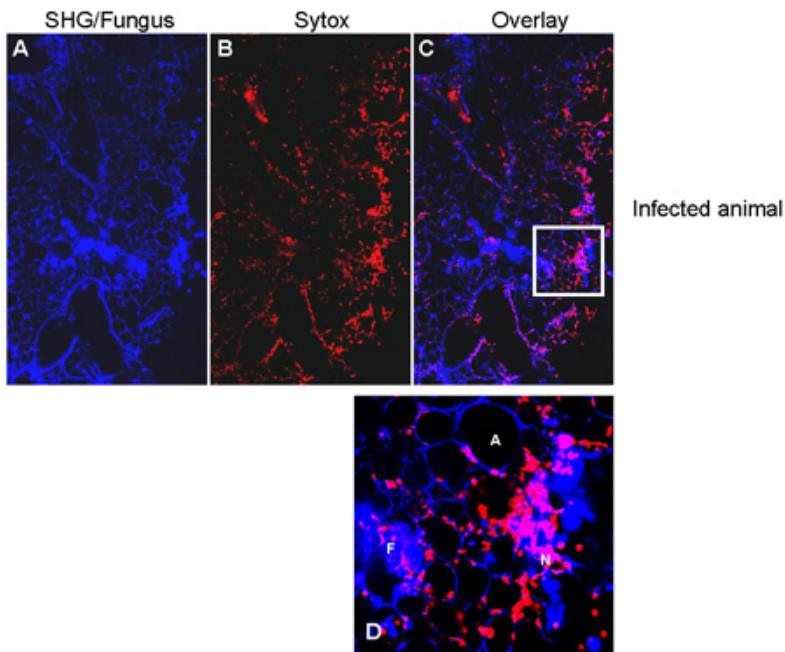


Figure 2. The appearance of a lung slice of an *Aspergillus* infected wild type animal in 2-color 2-photon microscopy. A lung slice from a C57/BL6 mouse infected 7 h before with *A. fumigatus* was prepared and imaged as described in the protocol. Shown is the combined fungal structure and SHG of the alveolar tissue (A), the Sytox signal of DNA NETs as well as cell nuclei cut open during the preparation of the lung slice (B), and an overlay of the two channels (C). The boxed area in (C) is seen enlarged in (D). Please note, the clearly distinct areas of fungal masses, alveoli, and NET-structures are marked with the letters F, A and N, respectively.

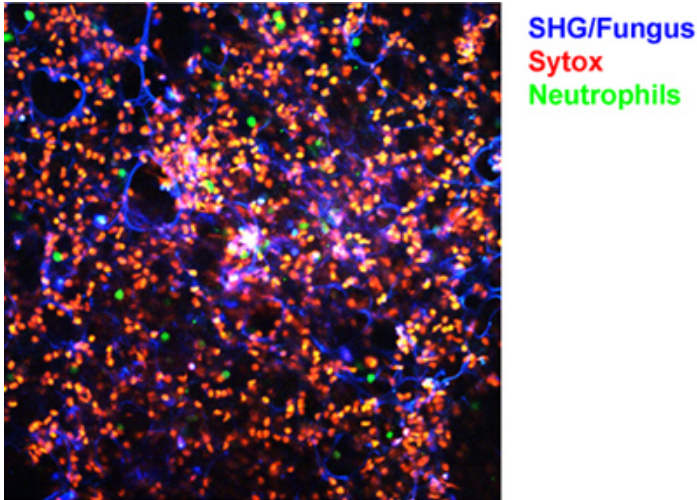
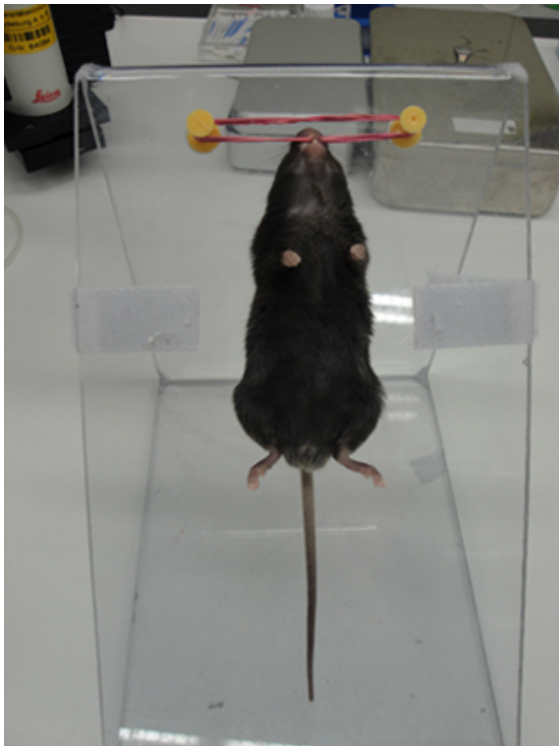
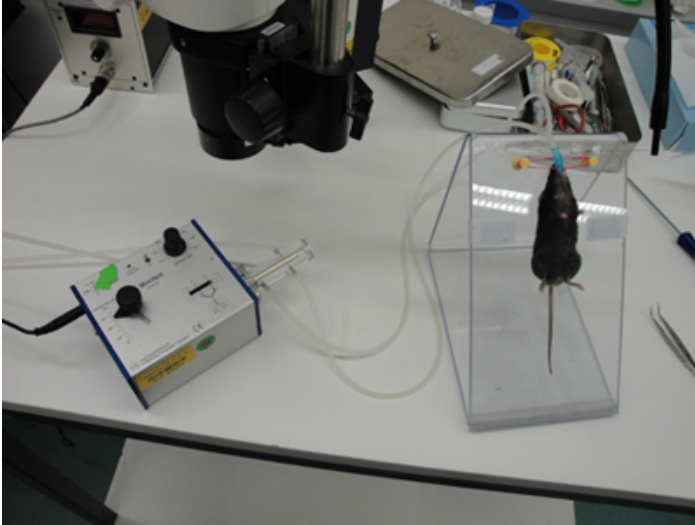


Figure 3. The appearance of a lung slice of an *Aspergillus* infected Lys-EGFP animal in 3-color 2-photon microscopy. A lung slice of a Lys-EGFP mouse infected 7 h before with *A. fumigatus* was prepared and imaged as described in the protocol. Shown is the combined fungal structure and SHG of the alveolar tissue (blue), the Sytox signal of cell nuclei and NETs (red), as well as well as numerous neutrophils (green).



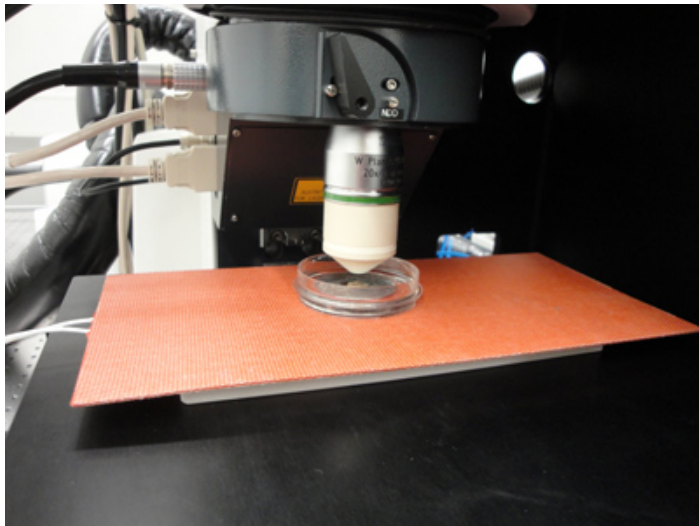
Supplemental figure 1. Mouse fixation for intubation. Under Ketamin/Rompun anesthesia the animal is fixed with an elastic band at its teeth in order to facilitate the intubation with a 22G indwelling venous catheter.



Supplemental figure 2. Mechanical mouse ventilation. The intubated mouse is infected by an i.t. application of 1×10^7 spores resuspended in $100 \mu\text{l}$ PBS. An enhanced distribution of fungal particles inside the lung is achieved by mechanically ventilating the infected mouse with a small animal respirator.



Supplemental figure 3. Lung lobe fixation. After preparation of the right lung lobe the organ is fixed in a Petri dish by use of a laboratory-made flat washer that is covered by a set of parallel nylon threads.



Supplemental figure 4. 2-photon imaging setup. The Petri dish containing the right lung lobe is installed on a heating mat under the 2-photon microscope after addition of the DNA dye Sytox Orange.

Supplemental movie: Neutrophils migrating and phagocytosing fungal elements in *Aspergillus* infected lungs as seen by time lapse 2-photon microscopy of a living lung slice 7 h after infection with the fungus. Neutrophils are green, fungal elements and SHG are blue, and cell nuclei as well as NET-structures are depicted in red. The real time of the experiment is shown on the lower right. The scale bar depicts 50 μm . [Click here to watch video](#)

Discussion

Real-time 2-photon microscopy *in vivo* or in intact organs has gained profound importance in studies dealing with the physiology of immune cells over the past 10 years. It was with this technique that important events like the dynamics of T-cell activation within the lymph nodes first became visible²⁻⁴. More recently, researchers have also started to analyze specific cellular functions like the first steps in the generation of effector cells in lymphatic tissues using this approach⁵.

However, although a number of new biological concepts have been revealed using this method, there are still challenging and important questions for which no intravital visualization studies have been published thus far. Notably this applies to the mammalian lung. The interesting aspect of this organ, as entry port for a variety of airborne pathogens, makes it one of the most crucial surfaces at which immunological processes take place in the mammalian body. With every breath over the entire life span, unwanted particles are inhaled some of which have the potential to induce life threatening infections⁶. It is self-explanatory that at such a sensitive and endangered site a tight network of defense mechanisms needs to be present exhibiting the whole repertoire of immune responses. On the other hand it is very important that the induced immunological fight against potential pathogens at such a "dirty" place is tightly controlled. Exaggerated reactions of the immune system bear a high risk of harming the own body by massively injuring organ tissue upon stimulation of unspecific immune cell actions^{7,8}.

In the light of these thoughts it would be extremely interesting and helpful to have the possibility to investigate cell behavior in mammalian lungs under true *in vivo* conditions. However, the fact that such a system has so far not been successfully implemented clearly points to the enormous difficulties that have to be solved in setting up a working protocol. The most demanding challenge probably is focus stability. The lung as the organ responsible for breathing is under constant movement in all three directions of space to realize inhalation. This circumstance alone causes severe imaging problems and can be considered an intravital imager's "nightmare". Already a slight motion to any dimension in space has a massive deteriorating effect on the microscopic view, which needs to be stable with micrometer precision in order to generate meaningful images⁹. Given its inherently tight local focus¹⁰, 2-photon microscopy is even more sensitive to focal instabilities, as a dislocation of a certain structure in the range of just a few micrometers in the Z-direction is equivalent to a complete loss of focus and thus a failed experiment.

The protocol presented in this study for the observation of immune cells within the murine lung is still not an *in vivo* application, but rather a close approximation to the situation in a functionally intact lung¹¹. *Ex vivo* approaches for imaging lymphocytes, e.g. in explanted lymph nodes, have been shown to yield results equivalent to true *in vivo* observations¹² and thus are highly relevant⁵. The *in situ* observations in lung slices, which are only possible with our approach, take place in an infected lung shortly after excision. The 3D integrity during the cutting process is ensured by the agarose matrix, an essential step to allow a precisely controlled cutting process of the lung. Although it is necessary to cool the explanted lung for a short period to allow a solidification of the agarose matrix, it is possible to return to near physiological conditions for cells after cutting and rewarming of the tissue. This is clearly shown by our data, which demonstrate that under these conditions neutrophils are highly active and exhibit their full potential as agile phagocytes, which are necessary for the effective clearance of an *Aspergillus fumigatus* infection. They patrol the lung tissue passing through epithelial barriers in order to reach the inner parts of alveoli and furthermore they actively take up fungal spores¹¹. A key finding of this work was the appearance of structures resembling Neutrophil extracellular traps (NETs) in the *Aspergillus* infected organ. NETs are a very recent finding of a novel defense mechanism in neutrophils¹³. However, since its initial description in 2004, the number of physiological or pathological conditions in animal models or humans where this phenomenon has been observed or is lacking has been explosively increasing¹⁴⁻¹⁶. Interestingly, although so much work has been spent on these structures by so many different groups, most reports are still on a very descriptive level and not much is known about the mechanism of NET release and its regulation. With our protocol we were able for the first time to show NET fibers in an infected lung. Furthermore, we could demonstrate the importance of freshly recruited neutrophils as well as molecular fungal structures for their occurrence or inhibition¹¹. This clearly shows the potential of our method to investigate

the single steps of NET formation in more detail. One could think for example to use neutrophils from suitable knock-out mice to observe their ability of NET formation in adoptive transfer experiments.

Thus, although the direct observation of neutrophil immigration from the peripheral blood is not possible with this system due to the lack of blood supply after organ explantation, we still believe that our protocol is a valuable and relatively easy to handle approach that allows the imaging of early or late steps in the immune defense against lung infections. This is, therefore, an important step towards investigating this phenomenon within the breathing lung of live animals.

Disclosures

We have nothing to disclose.

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