

Bismuth Ferrite Second Harmonic Nanoparticles for Pulmonary Macrophage Tracking

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Recently, second harmonic generation (SHG) nanomaterials have been generated that are efficiently employed in the classical (NIR) and extended (NIR-II) near infrared windows using a multiphoton microscope. The aim was to test bismuth ferrite harmonic nanoparticles (BFO-HNPs) for their ability to monitor pulmonary macrophages in mice. BFO-loaded MH-S macrophages are given intratracheally to healthy mice or BFO-HNPs are intranasally instilled in mice with allergic airway inflammation and lung sections of up to 100 μM are prepared. Using a two-photon-laser scanning microscope, it is shown that bright BFO-HNPs signals are detected from superficially localized cells as well as from deep within the lung tissue. BFO-HNPs are identified with an excellent signal-to-noise ratio and virtually no background signal. The SHG from the nanocrystals can be distinguished from the endogenous collagen-derived SHG around the blood vessels and bronchial structures. BFO-HNPs are primarily taken up by M2 alveolar macrophages *in vivo*. This SHG imaging approach provides novel information about the interaction of macrophages with cells and the extracellular matrix in lung disease as it is capable of visualizing and tracking NP-loaded cells at high resolution in thick tissues with minimal background fluorescence.

1. Introduction

Nanoparticles (NPs) increasingly govern the development of novel therapeutics and diagnostic enhancers. NPs can not only provide contrast but also allow tracking of cells and monitoring their distribution over time.^[1,2] Most commonly this has been accomplished in combination with optical imaging,

utilizing near-infrared NPs.^[3] While optical imaging can provide functional information, its main disadvantage is the low penetration depth and high background from tissue autofluorescence.^[4] More recently however, second and third harmonic generation (SHG and THG) nanomaterials have been generated that can be efficiently employed in the second near infrared window (NIR-II)^[5] using a multiphoton microscope. They have several inherent advantages, i) providing a significantly higher penetration depth; ii) no bleaching or blinking; and iii) emitting both, SHG and THG signals that allow them to be detected regardless of the endogenous background signal emitted by collagen and lipids for example.^[6,7] Bismuth ferrite (BiFeO_3) harmonic nanoparticles (BFO-HNPs) have excellent SHG and THG capabilities^[8] and were recently shown to be highly biocompatible in human cell lines,^[9] and can be used for monitoring of human stem

cells in depth of skeletal muscle tissue at more than one millimeter from the surface.^[6] BFO is the material of choice among all metal-oxides since its nonlinear coefficients is one of the highest, along with its large wavelength excitation range, reaching further into the infrared window.^[8]

In this work, we demonstrate the possibility of using BFO-HNPs for monitoring pulmonary macrophages. The lung

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is an attractive organ for the administration of novel targeted biomedical. Not only does it provide a vast surface of epithelial and vascular cells, which facilitates easy uptake of solutions and biomaterials, but it also presents the advantage of local delivery by intratracheal, inhalation, or intranasal application. Systemic delivery of therapies or diagnostic enhancers commonly struggles with off target or side effects, and in particular nanomaterials are frequently cleared too fast by the renal and hepatic system when applied to the blood circulation.^[10,11] By contrast, administration of NPs into the respiratory tract facilitates a prolonged persistence by direct interaction with the targeted cells, depending on particle size, charge, and surface modification.^[12] The uptake of NPs by immune cells is of particular interest as it could potentially have an immunomodulatory effect^[13,14] and influence the fate and thus the effect of inhalable nanoparticulate drugs. Moreover, lung diseases that are associated with inflammation, such as asthma or COPD, frequently demonstrate an increase in the number of immune cells such as macrophages which have been shown to be potential ideal carriers for diagnostic or therapeutic particles.^[1,3,15,16] On the other hand, NPs have also been reported to exacerbate inflammatory responses, which has led to the increased need of thorough evaluation of toxic effects of nanomaterials before their use for therapies.^[17]

Here we employ two-photon-laser scanning microscopy (2P-LSM) to show that bright SHG emission by BFO-HNPs can be localized and tracked in externally applied cells or in endogenous macrophages in 3D in thick lung tissue sections of mice, with minimal background and distinguishable from the intrinsic collagen signal. In mice with allergic airway inflammation (AAI), BFO-HNPs were detected in alveolar macrophages as long as 24 h after instillation, showing their stability and capability to remain intact once phagocytized by macrophages. We thus established a valuable optical imaging tool for tracking of immune cells in the lung, which enables the assessment of their biodistribution and potentially that of their activity and function.

2. Results

To assess the applicability of BFO-HNPs (**Figure 1A**) for imaging cells of the respiratory tract, we gradually tested the BFO-HNPs by starting with the *in vitro* uptake by MH-S macrophages in cell based assays, and then proceeding with *in vivo* applications by first instilling BFO-HNPs loaded alveolar MH-S macrophages intratracheally (i.t.) to the lungs of mice, and secondly by direct intranasal (i.n.) administration of serum-coated BFO-HNPs. We used suspensions of BFO-HNPs that were stable with values of zeta potential below -30 mV at pH 7. The mean particle size was previously estimated by dynamic light scattering (DLS) at about 100–120 nm, as also confirmed by scanning electron microscopy (SEM).^[8] Uncoated NPs were chosen, because they were earlier shown to be more efficiently taken up by macrophages.^[9]

BFO-HNPs are readily phagocytized by MH-S cells: To assess the uptake of BFO-HNPs by macrophages, MH-S cells were incubated with a freshly sonicated suspension of BFO-HNPs in cell culture medium and visualized by 2P-LSM after

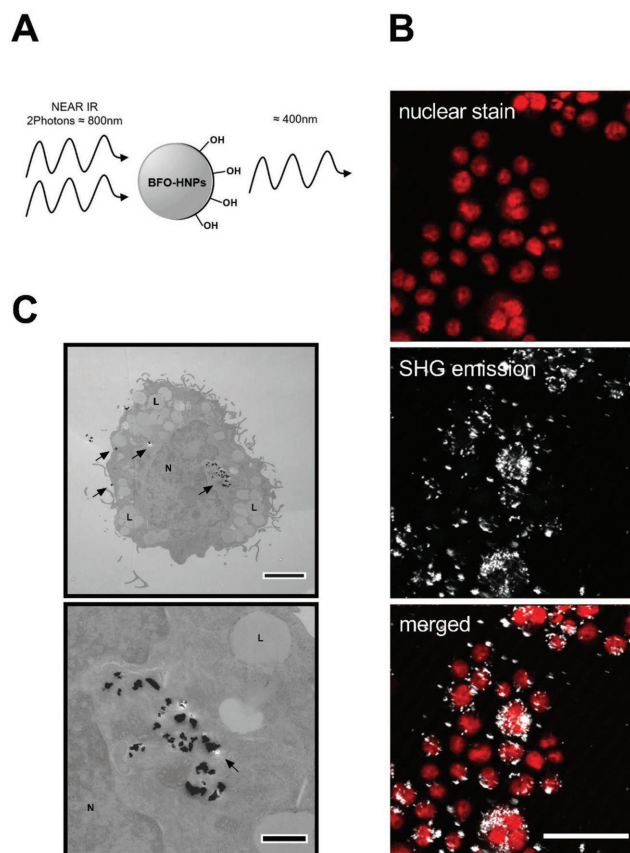


Figure 1. *In vitro* uptake of BFO-HNPs by MH-S cells. A) Scheme showing the characteristic harmonic features of BFO-HNPs. B) Representative 2P-LSM images of MH-S cells, *in vitro* cultured with BFO-HNPs for 24 h, showing their SHG emission (white signal) within the cells and their nuclei stained with DRAQ5 (red signal). Scale bar represents 50 μ m. C) EM images show BFO-HNPs in endosomes of MH-S cells after 24 h of incubation (arrows). L, lipid droplets; N, nucleus. Scale bars represent 5 μ m, inset 1 μ m.

5 h (data not shown) and 24 h (**Figure 1B**). BFO-HNPs were efficiently taken up by MH-S cells within the first 5 h of incubation, and after 24 h incubation no adverse effect, such as apoptosis was found (data not shown). As shown in **Figure 1B**, intense fluorescent spots are detectable in the cytoplasm of MH-S cells, which are most likely associated to small BFO-HNP aggregates formed upon uptake and internalization in the cells. To examine the intracellular localization of BFO-HNPs, we performed electron microscopy of BFO-HNP loaded MH-S cells. After 1 day of incubation with the cells, the detailed structure of the BFO-HNPs is shown as a NP with an irregular shape and strong electron density (**Figure 1C**). Some NPs were found adhering to the cell surface, while others appeared inside of the cells.

Those that were internalized appeared both, inside of endosomes and lysosomes (**Figure 1C**, arrows). They were never observed in the nucleus. These results point to an endocytic pathway for BFO-HNP internalization, consistent with earlier findings on BFO-HNPs^[6,9] and other nonligated NPs of similar size.^[18,19]

BFO-HNPs can be visualized in the context of lung tissue: To evaluate the SHG and THG capabilities of BFO-HNPs in lung tissue

and to assess the possibility of using BFO-HNP-based labeling to track cells of interest in the lung, we first tested the visualization of BFO-HNPs loaded MH-S cells that were applied i.t. into the lungs of healthy mice by means of 2P-LSM. Thick lung tissue slices of up to 60 μm were then used to visualize the BFO-HNPs. As can be seen in **Figure 2A**, BFO-HNPs were detected in superficially localized MH-S cells as well as deep inside the lung tissue mostly around bronchi and blood vessels 24 h after

i.t. application of the BFO-HNPs loaded MH-S cells. Excitation at 810 nm resulted in SHG signals, which correlate with the BFO-HNPs particles localized at the cell body region, around the nuclei of MH-S cells located in the lung tissue. This result confirms that the i.t. delivered MH-S cells remain intact in the lungs of healthy animals (**Figure 2B**, *F4/80 stain, green signal*).

The SHG emission is so spectrally narrow that when excitation wavelengths are set slightly out of the proper filter range

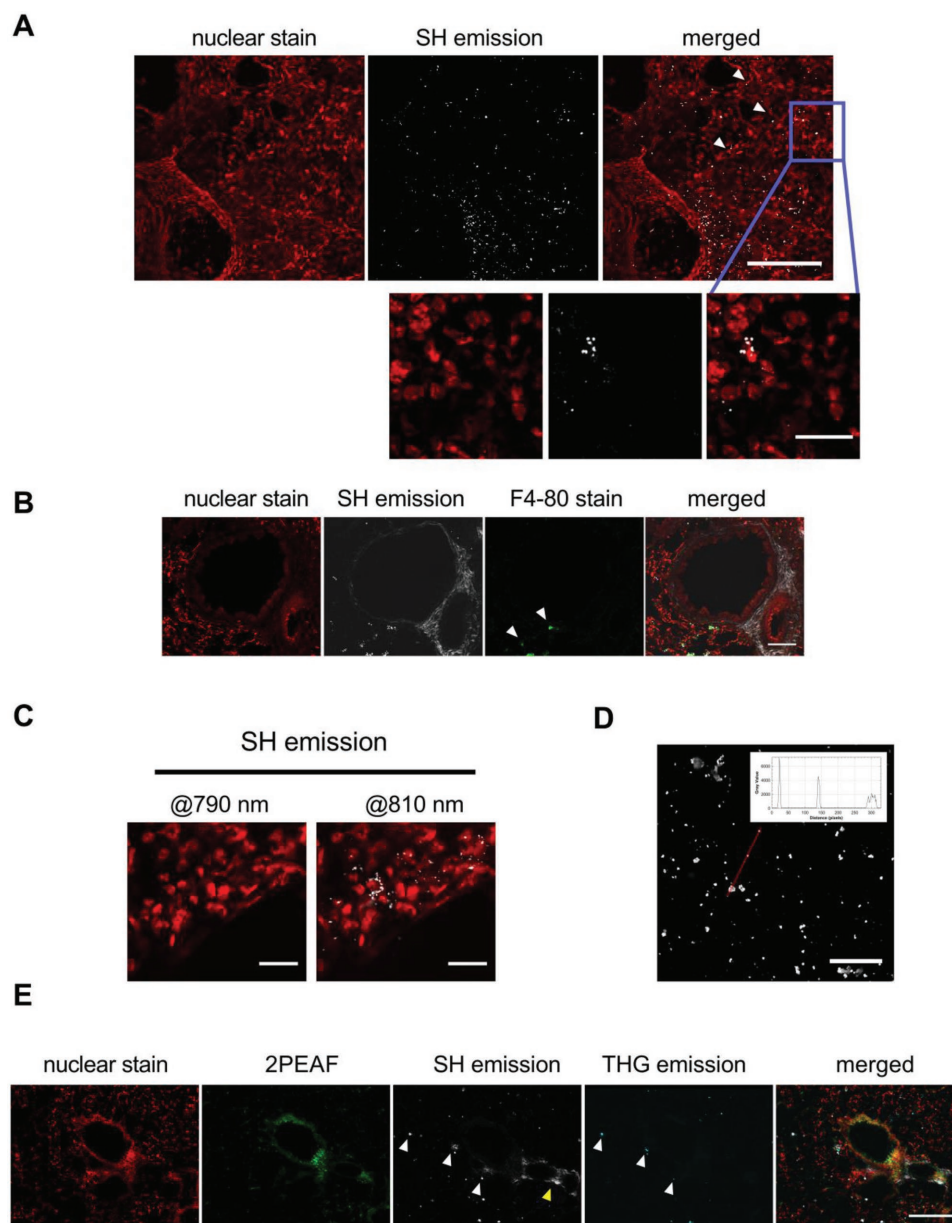


Figure 2. BFO-HNPs have excellent SHG and THG capabilities in lung tissue. Representative 2P-LSM images show: A) BFO-HNPs signals within MH-S cells (*white arrow heads*) located in the lung, 24 h after i.t. application of cells into healthy mice. Scale bar represents 100 μm . B) Stain for macrophage marker F4/80 (*green signal, white arrow heads*) shows the BFO-HNPs containing MH-S cells within the lung tissue in healthy mice. C) Nonlinear response of BFO-HNPs acquired by 2P-LSM at 790 nm (*left panel*) and 810 nm (*right panel*). Scale bar represents 25 μm . D) The graphic shows the signals obtained from the BFO-HNPs selected by the red line in the image and neglectable background signals. Scale bar represents 100 μm . E) Simultaneous emission of SHG (*white arrow heads*) and THG (*white arrow heads*) by BFO-HNPs within MH-S cells located in lung tissue. Two-photon excited auto-fluorescence (2PEAF) is also shown (*green signal*). The SH emission by collagen in the lung tissue is also shown (*yellow arrow head*). Scale bar represents 200 μm .

the signal was completely absent. This is demonstrated in Figure 2C, where even a difference of 20 nm (790 vs 810 nm) led to a loss of SHG detection through the 405 ± 5 nm filter. The measured signal-to-noise ratio (SNR) was as high as ≈ 8 dB. Figure 2D shows their excellent harmonic signals detected over the background signal at 810 nm. After multiple excitations, there was no loss of signal intensity (data not shown), showing one of the inherent advantages of these BFO-HNPs, i.e., no bleaching and blinking. Despite the two-photon excited auto-fluorescence (2PEAF, *green signal*) and the SHG signal emitted by collagen fibers (yellow arrow, *white signal*), the BFO-HNPs can still be detected by their simultaneous strong emission of SHG and THG signals (*cyan signal*), the latter not being produced by collagen (Figure 2E).

These results demonstrate that BFO-HNPs signals can be detected in ex vivo loaded and i.t. instilled MH-S cells and enable localizing these cells in lung tissue by their strong specific SHG signal. The detection of BFO-HNPs in MH-S cells as long as 24 h after instillation suggests a very good stability of these NPs and no loss in fluorescence intensity, in line with earlier findings about these NPs.^[7]

BFO-HNPs are phagocytized by lung macrophages: To visualize and track BFO-HNPs in the endogenous environment of the lung, we directly applied serum-coated BFO-HNPs i.n. to mice with AAI and to control animals. The induction scheme of the AAI model is shown in Figure 3A. Hematoxylin and eosin (H&E) staining of lung paraffin sections showed the typical infiltration of immune cells into the lung of AAI mice as opposed to controls, most prominent around the vessels and bronchial airways (Figure 3B). Bronchioalveolar lavages (BALs) from AAI mice contained around 20–45% of eosinophils (data not shown), similar to previously reported numbers with this ovalbumin (OVA)-induced AAI model.^[3,20] All mice that received serum-coated BFO-HNPs i.n. showed no adverse effects 24 h after i.n. application, indicating that BFO-HNPs are physiologically inert and nontoxic.

Immunofluorescence and nonlinear signals of agarose lung sections explanted 24 h after instillation of BFO-HNPs showed that the HNPs are located in alveolar and bronchial eosinophil chemotactic factor-L (ECF-L)-positive immune cells in both control and AAI lungs (Figure 4A,B; magnified images). ECF-L is

a marker used to identify M2 macrophages ($M\Phi$), abundantly expressed in allergic inflammation. Stitched mosaic images of AAI lung sections show significantly higher numbers of ECF-L⁺ cells containing BFO-HNPs around bronchia and blood vessels (Figure 4B) than those of control lungs, where the cells are fewer and more spread around the tissue (Figure 4A). AAI sections also presented more SH signal from collagen, which may suggest that lungs from AAI mice exhibit more extracellular matrix than control lungs. Lungs of AAI animals commonly present regions of dense immune cells infiltration, mostly around bronchial structures. These regions never showed any BFO-HNPs within immune cells, similar to what we found previously with other NPs in this AAI model.^[3] The significance of this observation still has to be explored in future studies.

The simultaneous detection of collagen and BFO-HNPs containing cells is also visualized in 40 μ m agarose lung sections (Figure 5). We show a 3D lung section where we can visualize the collagen fiber network (Figure 5A,B, *yellow arrow head*) around blood vessels and bronchia through the whole section and still precisely identify the macrophages that contain BFO-HNPs (Figure 5A,B, *white arrow heads*).

This is possible because NIR light is used to generate SH emission by the BFO-HNPs, which allows a deeper penetration into the tissue. In this example, it is easily possible to specifically separate the SHG signals of the collagen fibers from the signals produced by the BFO-HNPs because of their spatial and morphological differences. In tissue sections where this is not the case, i.e., SHG signals from collagen and NPs are too close to each other, the THG capabilities of the BFO-HNPs can be used to distinguish them from the SHG signal of collagen, as showed in Figure 2D.^[6,8]

BFO-HNPs are present in endogenous alveolar macrophages: We then went on to identify if there was a selective uptake of the BFO-HNPs depending on the macrophage type upon i.n. application of the HNPs into AAI mice. To this end, we performed several immunofluorescent stainings with a number of antibodies directed to proteins that are known to be expressed on macrophages. We found that BFO-HNPs were localized exclusively in CD68⁺, F4/80⁺, ECF-L⁺, and major histocompatibility complex class II (MHCII⁺) cells on

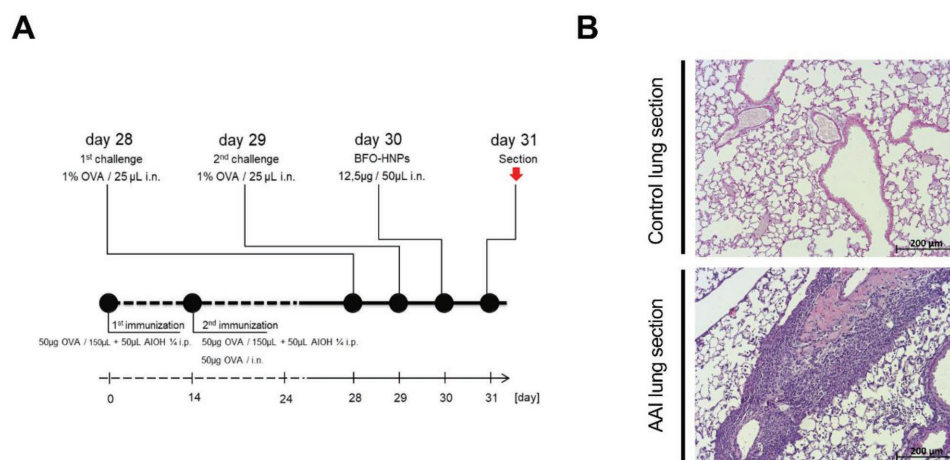


Figure 3. Mouse model of AAI. A) The immunization and challenge scheme for the AAI model used in all investigations; B) H&E staining of paraffin sections from lungs shows strong infiltration of immune cells in AAI mice as compared to controls. Scale bar represents 200 μ m.

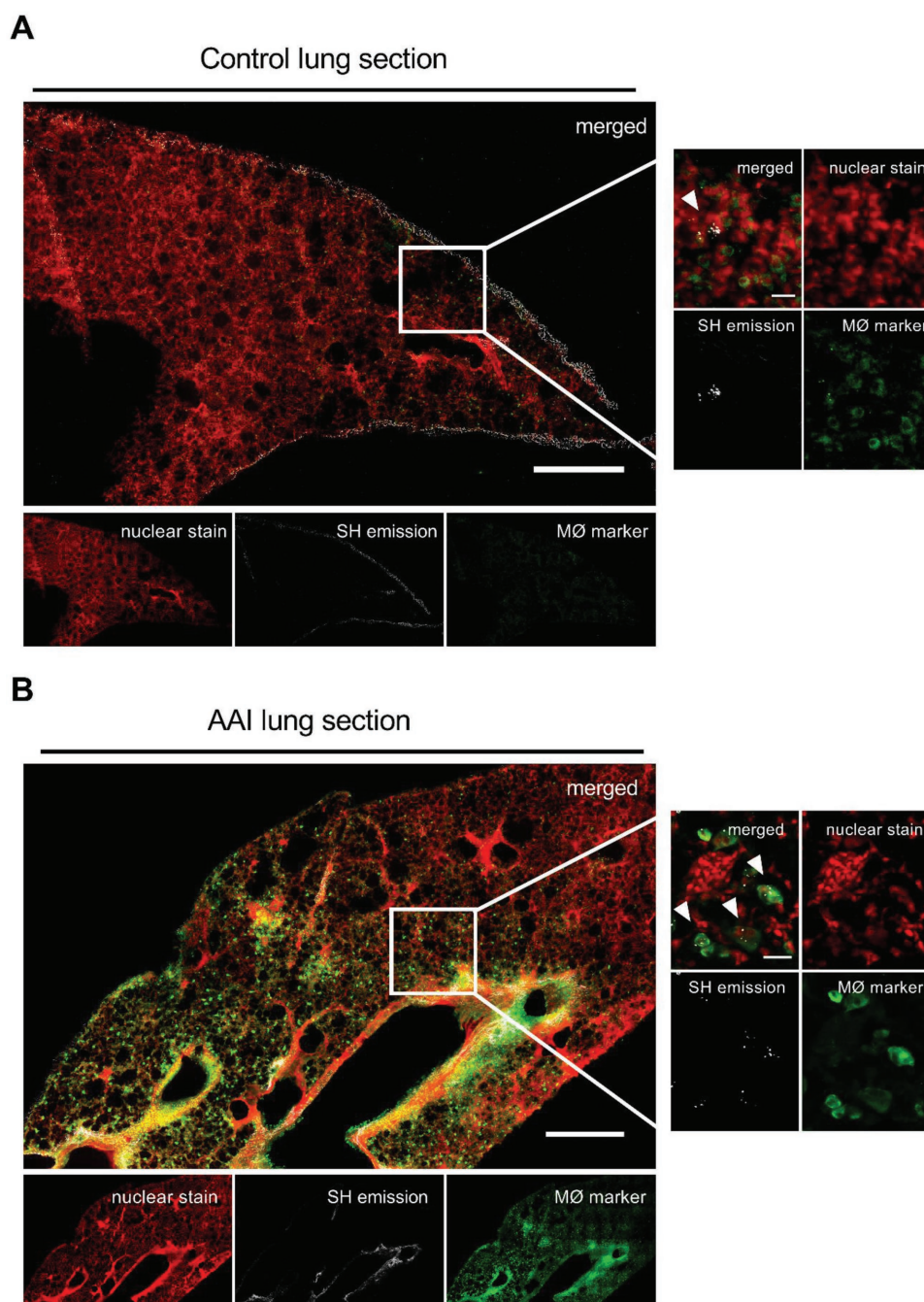


Figure 4. Serum-covered BFO-HNPs are readily phagocytosed by native macrophages. A,B) Representative 2P-LSM images of 40 μm lung tissue sections from AAI and control mice stained for the M2 macrophage marker ECF-L (M Φ). The stitched images of A) 11 $\times$ 7 tiles (350 $\times$ 350 μm each) in control and B) 13 $\times$ 18 tiles (336.91 $\times$ 336.91 μm each) in AAI lungs show significantly more BFO-HNPs containing cells in the AAI lung section (A,B, zoomed images, *white arrow heads*). SH emission from collagen is also visible (*white signals*). DRAQ5 was used as nuclear stain in all images. Scale bar represents 500 μm , and in the zoomed pictures 20 μm .

thick agarose sections of lung tissue (Figure 6) or cytopins from BALs (Figure 7), identifying them as M2 alveolar macrophages.

One of the expected clearance pathways of NPs from the respiratory tract following the deposition of particles into the tracheobronchial region is their removal through pulmonary alveolar macrophages by interstitial route.^[21–23]

These pulmonary alveolar macrophages can be easily isolated through BALs. We found that most of the cells from the BALs of AAI mice were composed of macrophages, which contained BFO-HNPs and were CD68⁺, ECF-L⁺, and MHCII⁺ (Figure 7). This suggests that M2-activated alveolar macrophages take up BFO-HNPs, in line with previous reported pathway of clearance in an inflammatory environment.^[3]

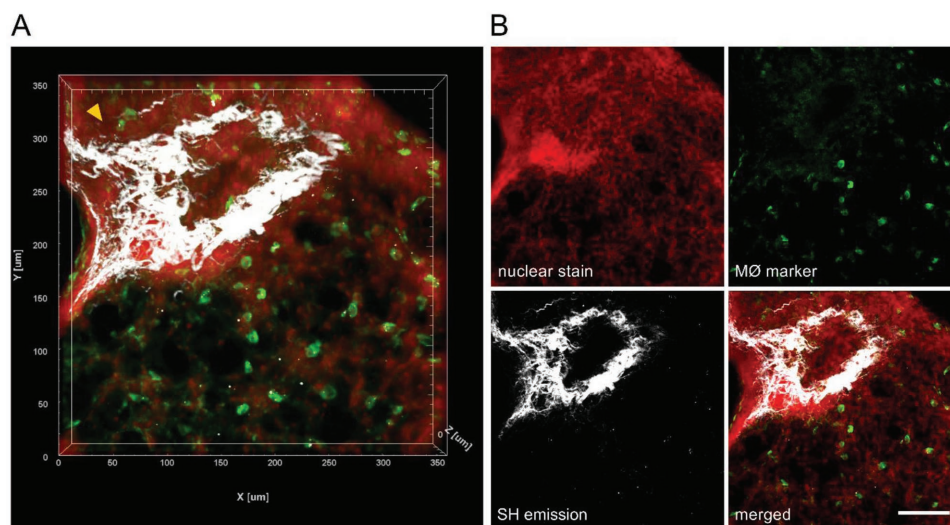


Figure 5. BFO-HNPs are visualized in collagen-rich tissue. A) 2P-LSM images show that i.n. instilled BFO-HNPs localized in macrophages (green signals) are visualized in 40 μm thick lung section of AAI mice between collagen-rich regions, most likely around a blood vessel (yellow arrow head). B) The SH emission of collagen and as well from the BFO-HNPs (white signal) and the macrophage marker ECF-L (green signal) localized in the middle of the section. Nuclei were stained with DRAQ5 (red signal). Scale bar represents 100 μm .

3. Discussion

Novel nanoparticulate materials can enormously advance diagnostic and therapeutic approaches. SHG capable NPs are of particular interest due to their many advantages over conventional fluorescent materials. The choice of such a nanomaterial for this study is based on the need to maximize imaging depth

and maintain high selectivity to endogenous tissue emission. We show that BFO-HNPs perfectly fit these requirements by stably labeling exogenous or endogenous macrophages and producing sufficient SH signal to be detected after their in vivo application to mice, providing the possibility to track these macrophages and visualize them in the context of neighboring tissue structures.

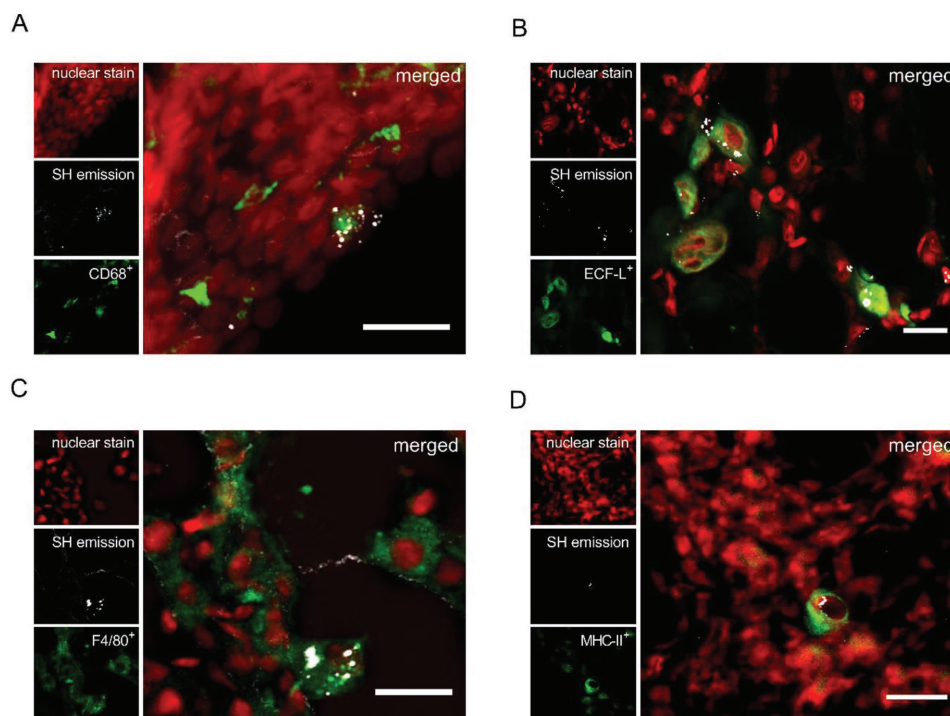


Figure 6. BFO-HNPs are phagocytized by endogenous lung tissue alveolar macrophages. 2P-LSM images show that i.n. instilled BFO-HNPs (white signals) are localized in cells positive for A) CD68, B) ECF-L, C) F4/80, and D) MHC-II (green signals) in agarose lung sections of AAI mice. Nuclei are stained with DRAQ5 (red signal). Scale bar represents 20 μm .

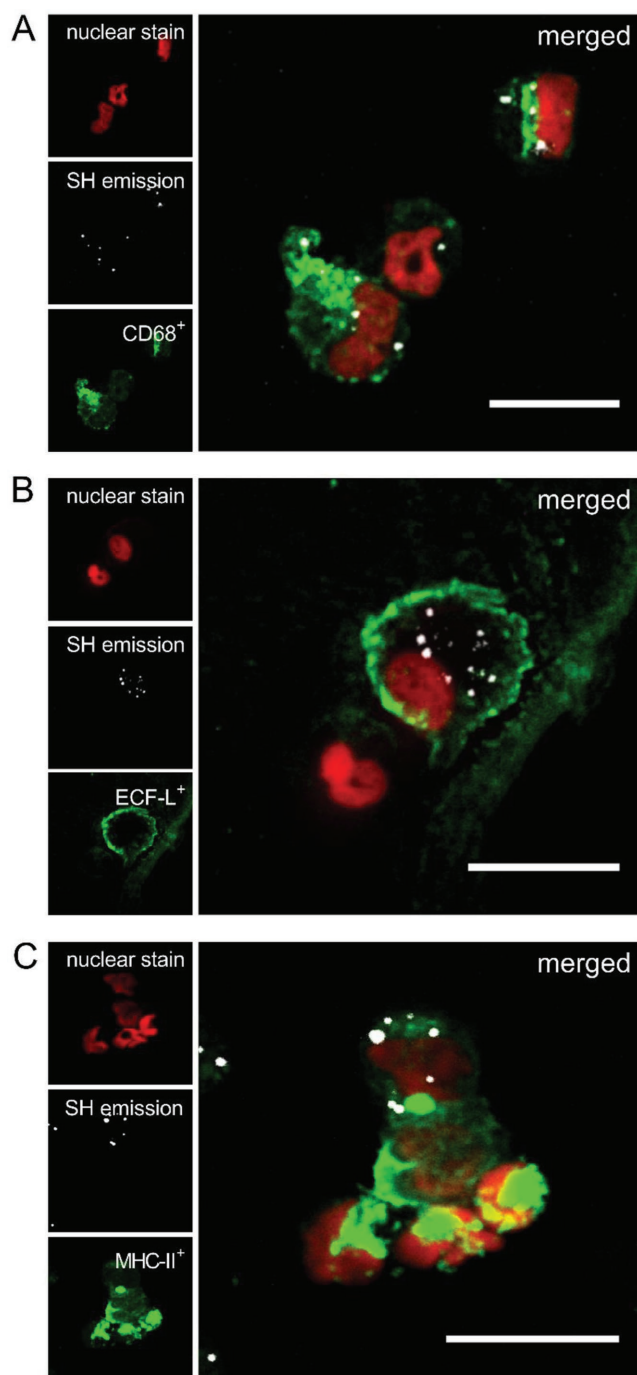


Figure 7. BFO-HNPs are phagocytized by endogenous alveolar macrophages obtained by BAL. 2P-LSM images show that i.n. instilled BFO-HNPs (white signals) are localized in cells positive for A) CD68, B) ECF-L, and C) MHC-II (green signals) from cytopspins of BALs of AAI mice. Nuclei were stained with DRAQ5 (red signal). Scale bars represent 20 μm .

In *in vitro* fluorescence and EM experiments, we could show that these nanomaterials are efficiently taken up by macrophage derived cells, where they enter the cells through the endocytic pathway and accumulate in the perinuclear location

inside of endosomes or lysosomes, consistent with previous reports.^[19,24]

We show that bright BFO-HNP signals could be detected within the lung by 2P-LSM from MH-S cells that were *ex vivo* loaded with BFO-HNPs and then instilled i.t. into healthy mice. This suggests that the cells are stably labeled with these NPs allowing them to be tracked for at least 24 h. We have found similar results earlier with barium-sulfate loaded MH-S cells also applied i.t., which were found in alveoli and bronchial airways using synchrotron radiation inline free propagation phase contrast computed tomography.^[1] As previously reported by our group, the i.t. inhalation of macrophages loaded with barium NPs produces highest intensity signals in the lung, and almost no signal was detected outside this organ.^[1] Directly i.n. applied BFO-HNPs to AAI and control mice revealed an efficient uptake of the NPs by endogenous cells in the bronchial and alveolar system after 24 h. In both models—i.t. applied BFO-HNP loaded cells or directly i.n. applied BFO-HNPs—the nanocrystals were detectable in cells that were located either superficially or deep within the lung tissue. Using a narrow emission filter, BFO-HNPs could be identified in the lung with an excellent SNR of ≈ 8 dB and neglectable background signal. Since the BFO-HNPs also provide a THG signal, the nanocrystals can be easily differentiated from the endogenous SHG from lung collagen around the blood vessels, as already described.^[6,7]

We found that directly i.n. applied BFO-HNPs were primarily taken up by endogenous alveolar M2 macrophages resident in the alveolar spaces, in line with many reports of other NPs of similar size (100–200 nm) that are applied to the respiratory tract.^[3,25] We also found a substantially larger amount of BFO-HNP-labeled M Φ in AAI lungs compared to control lungs, consistent with our earlier findings of NIRF-labeled NPs in this AAI model.^[3] Recently M2 polarization was shown to enhance silica nanoparticle uptake by macrophages.^[26] This could well be the case for the uptake of BFO-HNPs in our study too. Our results thus show that BFO-HNPs enable the *ex vivo* 3D investigation to obtain major information on the behavior of macrophages in the healthy and diseased lung, considering their localization in relation to blood vessels and bronchioles or the interactions with resident and circulating immune cells. In addition, they offer the possibility for *in vivo* tracking of externally applied BFO-HNP loaded macrophages in the lung, allowing precise localization of the cells and monitoring their distribution over time. A similar approach has been shown earlier for the tracking of BFO-HNP labeled human stem cells that were injected directly into the muscle of mice,^[6] where 24 h later the BFO-HNP labeled cells were still detectable. We went further with a more physiological method, by instilling the BFO-HNPs directly into the lungs of AAI and control mice. This method allows the tracking of endogenous immune cells that take up these NPs. *In vivo* lung endoscopy could be a realistic approach to follow BFO-HNP loaded immune cells long term by using intravital microscopy. The ability to comprehensively image the epithelium and alveolar spaces throughout an entire segment of the lung can provide additional information about the spatial distribution and interaction of macrophages, which is more difficult to appreciate in thin tissue sections. As the role of macrophages in pulmonary inflammation

is still controversially discussed,^[25,27] tracking of alveolar macrophages by means of BFO-HNPs may support the understanding of the role these cells have in the clearance of NPs as well as in the immunological response to inflammatory stimuli. As NPs are increasingly considered as potential drug delivery systems, SHG capable NPs such as the BFO-HNPs could be conjugated to drugs and may be preclinically employed to study their precise delivery in 3D in deep tissue. This may be particularly appealing for the potential targeting of macrophages that are actively involved in deleterious inflammation as is the case for tumor associated macrophages.^[28] In this respect, it is of note that PEGylated and uncoated BFO-HNPs were well tolerated by MH-S cells and other cell types used before.^[9]

The BFO-HNPs showed excellent long-term photostability when imaged in vivo, because of their nonlinear parametric interaction with the excitation field, implying no light absorption by the NPs. Similar approaches based on SHG generation could also be tested with other nanoprobe such as metal NPs^[29] and 2D transition metal dichalcogenides.^[30,31] Such a property is essential to perform tracking over days or weeks and represents a powerful tool for tracing macrophages in the entire lung as well as providing information on the fate of inhaled NPs. Finally, BFO-HNPs excitation wavelength flexibility widens the palette of molecular probes that can be used for fluorescence staining of structures of interest, as their narrow emission peaks can always be spectrally isolated by simply tuning the laser wavelength outside the fluorophores' absorption region.

4. Conclusion

In conclusion, we provide a promising approach to track macrophages in their endogenous environment with stable and intense SHG and THG signals from harmonic BFO nanocrystals. Future improvement of the visualization of even deeper tissue segments would be possible by longer wavelength excitation. These would enable the exploration of SHG, THG, and even fourth harmonic generation (FHG) signals produced intrinsically by tissue structures, minimizing the need of tissue labeling.

5. Experimental Section

Materials, Cells, and NPs Preparation: Uncoated BFO-HNPs were prepared according to the method described by Schwung et al.,^[8] and suspended in solution at 5 mg mL⁻¹ in 100% ethanol. DLS and SEM were performed as previously described.^[8] BFO-HNPs were always ultrasonicated for 30 min before each experiment to achieve optimum resuspension. Cells of the immortalized and adherent mouse alveolar macrophage MH-S cell line (CRL-2019, ATCC)^[32] were grown in complete RPMI medium, supplemented with 10% fetal calf serum (FCS) and 0.05 mM 2-mercaptoethanol.

For the in vitro experiments, i) 1×10^6 MH-S cells were plated into 25 mm Poly-L-Lysin coated coverslips, BFO-HNPs (1.25 $\mu\text{g mL}^{-1}$) were added for 5 h or 24 h at 37 °C, washed twice with phosphate-buffered saline (PBS) and then fixed at room temperature for 10 min with 10% formalin for 2P-LSM; or ii) 5×10^5 MH-S cells were plated into 3 cm Petri dishes (Nunc), BFO-HNPs (1.25 $\mu\text{g mL}^{-1}$) were added for 24 h at 37 °C, washed twice with PBS and then fixed with 2.5% glutaraldehyde overnight at 4 °C for EM.

For in vivo application of MH-S cells, 6×10^6 MH-S cells per well were grown in a 6-well plate and incubated with 360 $\mu\text{g mL}^{-1}$ of BFO-HNPs at 37 °C for 4 h, washed twice with PBS and subsequently each pellet per well was resuspended in 50 μL PBS before application to healthy mice.

For in vivo application of BFO-HNPs, the NPs were first ultrasonicated for 20 min, followed by dilution in 100% mouse serum (Immuno BioScience, 1/20 dilution rate) to a final concentration of 250 $\mu\text{g mL}^{-1}$, and again ultrasonicated for a further 1.5 h.

Induction of AAI: Pathogen-free, female BALB/c mice, 6–8 weeks of age, were purchased from Charles River Laboratories Inc. All animals were housed in a controlled environment at a regular 12 h dark:light cycle, at 22 °C and were fed laboratory chow and tap water ad libitum.

For induction of AAI, mice were immunized on days 0 and 14 via intraperitoneal (i.p.) injection of 50 μg OVA (Hyglos) and 0.5% aluminum hydroxide gel adjuvant (Invivogen) in a volume of 0.2 mL PBS, as well as i.n. with 50 μg OVA in 25 μL of PBS on day 14. On days 28 and 29 post immunization, mice were challenged i.n. with 250 μg OVA in 25 μL PBS to induce an acute allergic reaction. Control mice were immunized with PBS instead of OVA.

Application of NPs or NP-Loaded MH-S Cells: For the in vivo experiments, either i) 6×10^6 BFO-HNP loaded MH-S cells were applied i.t. to healthy mice or ii) a suspension of 50 μL of serum-coated BFO-HNPs (12.5 μg) was directly instilled i.n. into AAI animals and their respective controls 1 d after the last challenge.

For all i.n. applications, mice were briefly anesthetized by inhalation of 2% isoflurane, 2 L min⁻¹ oxygen. For i.t. instillation of the cells, mice were anesthetized with an i.p. injection of 15 mg kg⁻¹ BW xylazine (Ecuphar) and 75 mg kg⁻¹ BW ketamine (Medistar). Mice were sacrificed 24 h after i.t. or i.n. application of cells or BFO-HNPs and lungs were lavaged and/or harvested for further use. All animal experiments were performed in accordance with German animal ethics regulations and were approved by the local ethics office (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, LAVES, ethics approval no. 33.12-42502-04-12/0834).

Bronchoalveolar Lavage (BAL): BAL was performed by washing the airways gently three times with 500 μL of 2% FCS/PBS after exposing and cannulating the trachea. Volumes were pooled and then washed once in the same buffer. Recovered cells were counted in a hemocytometer and 3×10^4 cells were used for obtaining cytopspins with a Shandon Cytospin 4 (Thermo Scientific), followed by Giemsa staining (Sigma Aldrich) for differential cell counting. Where indicated, cytopspins were fixed with 1:1 acetone/methanol solution, immunostained, and counterstained with DRAQ5 (final concentration of 5 μM) for visualization of nuclei.

Immunofluorescence Staining and Histology: Explanted lungs were fixed overnight in 10% formalin. The samples were either embedded in paraffin or in 5% agarose blocks for preparation of serial lung sections. Two micrometers paraffin tissue sections were stained with H&E and inspected by routine light microscopy to assess the degree of cell infiltration in AAI mice and controls.

Agarose sections up to 60 μm were obtained with a Vibratome (Leica VT1000S) and washed three times in tris-buffered saline-Tween20 (TBS-T). The tissue slices were incubated with 3% hydrogen peroxide inhibitor (DAKO), to quench the endogenous peroxidases, and washed once again in TBS-T. Nonspecific binding was blocked by incubating the sections for 1 h in 2% bovine serum albumin in TBS-T. For tissue labeling, the agarose tissue sections were incubated free-floating in 500 μL of diluted primary antibody, followed by the same volume of secondary antibody. The following primary and secondary antibodies were used: rat-anti-mouse CD68 antibody (clone FA-11, Abcam, 1:300), followed by incubation with goat-anti-rat antibody Alexa Fluor 546 (Life Technologies, 1:400); Alexa Fluor 488 conjugated anti-mouse CD11c (clone N418, BioLegend, 1:200); Alexa Fluor 488 conjugated rat-anti-mouse MHCII antibody (clone M5/114.15.2, Biolegend, 1:200); polyclonal goat anti-mouse ECF-L antibody (R&D, 1:150), followed by donkey anti-goat-Alexa Fluor 546 secondary antibody (Abcam, 1:400), and rat-anti-mouse F4/80 (clone CL:A3-1, Serotec, 1:100) followed by goat-anti-rat Alexa Fluor 546 (Life Technologies, 1:400). Nuclear staining

was performed with DRAQ5 (Biostatus, 1:1000) and tissue sections were mounted using Cytoseal (Thermo Fisher Scientific).

Immunostaining of the cytopins prepared from the BALs were washed two times in Tris-buffer and nonspecific binding was blocked by incubating the cytopin slides for 1 h in sea block blocking buffer (Thermo Fisher Scientific). For immuno labeling, the cytopin slides were incubated overnight, at 4 °C, in a solution of diluted primary antibody (antibody diluent Dako), followed by 1 h incubation with the same volume of secondary antibody. The following primary and secondary antibodies were used: rat-anti-mouse CD68 antibody (clone FA-11, Abcam, 1:300), followed by incubation with goat-anti-rat antibody Alexa Fluor 546 (Life Technologies, 1:400); polyclonal goat-anti-mouse ECF-L antibody (R&D, 1:150), followed by donkey anti-goat-Alexa Fluor 546 secondary antibody (Abcam, 1:400), and the directly coupled Alexa Fluor 488 conjugated rat-anti-mouse MHCII antibody (clone M5/114.15.2, Biolegend, 1:200). Nuclear staining was performed with DRAQ5 (Biostatus, 1:1000), and cytopins slides were mounted using Cytoseal (Thermo Fisher Scientific).

Two-Photon Microscopy: Images were acquired with a 2P-LSM (TRiMScope II, LaVision BioTec) equipped with i) a femtosecond-pulsed titanium-sapphire (Ti:Sa) laser (Chameleon Ultra II; Coherent); ii) a 1300 nm femtosecond-pulsed ytterbium fiber laser with a fiber-based frequency conversion unit (Orange, Menlo Systems). A Zeiss W Plan Apochromat 20x (NA 1.0) water immersion objective was used for acquiring the images. For excitation of the SHG of the BFO-HNPs, the Ti:Sa laser was set at 810 ± 5 nm, and THG was produced by excitation at 1300 nm with the ytterbium fiber laser. Emitted light was split by a 495 and a 560 nm longpass dichroic mirror (Semrock) and collected by photomultiplier detectors (Hamamatsu) in either back- or forward direction. The light was collected through the following bandpass filters: a 405 ± 5 nm (FF01-405/10) for SHG, 435 ± 40 nm for THG, and a 525 ± 50 nm (FF01-525/50), respectively (Semrock). All images were collected and processed with ImSpector (LaVision Biotech), Fiji (Image processing package based on ImageJ), and Imaris 9.1.2 (Bitplane).^[33]

Electron Microscopy: MH-S cells were incubated with BFO-HNPs for 24 h and fixed with 4% formaldehyde and 2.5% glutaraldehyde in 0.1 M PBS pH 7.3. For embedding, the adherent cultures were treated on the culture dish with 1% OsO₄ (Science Services) in 0.1 M PBS and embedded in EPON (Serva) after dehydration with ethanol and en-bloc staining with 1.5% uranyl acetate (Merck) and 1.5% tungstophosphoric acid (Merck) in 70% ethanol. Ultrathin sections of cultured cells were cut parallel to the substrate using an UC7 Ultramicrotome (Leica) and stained with an aqueous solution of 4% uranyl acetate. Sections were viewed with a LEO EM912 Omega electron microscope (Zeiss) and digital micrographs were obtained with an on-axis 2k-CCD camera (TRS).

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

macrophages, nanoparticles, second harmonic generation, third harmonic generation, two-photon-laser scanning microscopy

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