Simultaneous Multi-Harmonic Imaging of Nanoparticles in Tissues for Increased Selectivity

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Abstract

We investigate the use of Bismuth Ferrite (BFO) nanoparticles for tumor tissue labelling in combination with infrared multi-photon excitation at 1250 nm. We report the efficient and
simultaneous generation of second and third harmonic by the nanoparticles. On this basis, we set up a novel imaging protocol based on the co-localization of the two harmonic signals and demonstrate its benefits in terms of increased selectivity against endogenous background sources in tissue samples. Finally, we discuss the potential use of BFO nanoparticles as mapping reference structures for correlative light-electron microscopy.

The advent of multi-photon microscopy in the early nineties has revolutionized the field of optical imaging. This technique has proven particularly beneficial for biological studies. Now a new milestone has been reached, thanks to the availability of compact ultrafast sources exceeding the traditional 700-1000 nm tuning range of Ti:Sapphire oscillators and covering the spectral region up to 1300 nm. Because of reduced scattering and photo-interaction with living matter in this wavelength region, these lasers open the way to improved performances in terms of imaging penetration enabling novel applications for multi-photon tissue diagnostics. On the other hand, traditional nanophotonics labelling approaches (quantum dots, plasmonic nanoparticles (NPs), up-conversion NPs) display fixed optical properties often in the UV-visible spectral region and cannot fully take advantage of this spectral extension. To circumvent wavelength limitations, a few research groups in the last years have introduced a new nanotechnological approach based on inorganic nanocrystals with non centrosymmetric lattice, harmonic nanoparticles (HNPs). By their crystalline structure, HNPs present very efficient nonlinear $\chi^{(2)}$ response, and can be effectively imaged using second harmonic (SH) emission as contrast mechanism. Being smaller than coherence wavelength, no phase-matching limitations apply and they efficiently respond to excitation from the UV to the mid-IR. Moreover their signal is not bleaching, blinking, nor saturating because of the non-resonant character of the photo-interaction mechanism.

In this work, we use Bismuth Ferrite (BFO) HNPs with a PEG biocompatible coating prepared for further functionalization. BFO HNPs have been recently presented as the most promising candidates for translating HNPs to medical applications. In fact, they present a very high second order nonlinear coefficient $\langle d \rangle = 79$ pm/V (for comparison $\langle d \rangle \approx 4$ pm/V for BaTiO$_3$ and KNbO$_3$ HNPs) and extremely good biocompatibility, in particular when PEG-coated. Cytotoxicity has...
been screened through various assays including high throughput methods. Recently, BFO HNPs have been applied with success to novel *in vitro* applications in cancer research and regenerative medicine.

Figure 1: Bare BFO HNPs deposited on a substrate. SH: second harmonic image; TH: third harmonic image; Scatter plot: TH vs. SH pixel intensity. The blue points show the co-localized events; SH∩TH: co-localization image.

For optical nonlinear imaging we employed a Nikon A1R multiphoton upright microscope (NIE-Nikon) coupled with an Insight Deepsee tunable laser oscillator (Spectra-Physics, 120 fs, 80 MHz, 680 - 1300 nm). The nonlinear signals were epi-collected by a Nikon 25× water immersion objective (CFI75 APO, N.A.1.1) spectrally filtered by tailored pairs of dichroic mirrors and interference filters and acquired in parallel either by a normal photomultiplier (600 - 655 nm) or a GaAsP photomultiplier (385 - 492 nm).

The top-left plot in Fig. 1 shows the SH emission at 625 nm generated by BFO HNPs deposited on a substrate when excited at 1250 nm. A part from the trivial case of large aggregates, the intensity differences among HNPs in the image reflect their size dispersion and crystal axis orientation.
with respect to the polarization of the excitation laser.\textsuperscript{13} Very interestingly, when detecting at the TH frequency (416 nm), HNPs appear also very bright. By applying an automatic co-localization algorithm developed by Costes et al. for fluorescent probes,\textsuperscript{14} we obtain more than 90% pixels co-localized, indicating that practically every particle on the substrate emits simultaneously at both harmonics. This result is clearly indicated by the scatter plot, where basically all events are included in the co-localization region defined by the black lines, and evident inspecting the co-localization image (SH∩TH), which bears no difference with respect to the corresponding SH and TH results.

By calibrating the spectral transmission of optics and response of the detectors, we could estimate the SH/TH BFO intensity ratio to roughly a factor hundred at 1250 nm and for 150 GW/cm\textsuperscript{2} peak pulse intensity at the sample. This intensity (calculated in the femtosecond regime and corresponding to 16 mW average power) is suitable for imaging of biological samples as it is sensibly lower than cell damage threshold established for visible wavelengths.\textsuperscript{15} Multi-harmonic emission of HNPs has been already sparsely reported.\textsuperscript{4,16,17} Our group has firstly observed it in Fe(IO\textsubscript{3})\textsubscript{3} HNPs by exciting at 1.5 μm, leading to SH intensity 100 times larger than TH.\textsuperscript{4} The normalized power dependence measured on a single HNP for both SH and TH with the characteristic $I^2$ and $I^3$ dependence is reported in Fig. 2a with the corresponding exponential fit confirming their assignment (1.95 and 3.2 for SH and TH, respectively). Clearly the SH/TH ratio changes according to excitation intensity and indeed Dai et al. have recently investigated this dependence in ZnO NPs for prospective applications in display technology.\textsuperscript{17} The same authors correctly pointed out that the use of longer wavelengths allows increasing excitation intensity concurrently maintaining (multi-photon) absorption at low levels, preserving sample integrity. In Fig. 2b, we show how the nonlinear axial point spread functions (PSF) measured at the second and third order on a single sub-diffraction limited HNP are different. Because of higher nonlinearity, TH PSF is in fact sensibly narrower, leading to increased resolution, an aspect that turns out to be particularly beneficial when working at long wavelengths.

Although a large $\chi^{(3)}$ response is not surprising for large $\chi^{(2)}$ samples,\textsuperscript{18} the simultaneous
Figure 2: A. Power dependence of the emission of a single BFO HNP measured at SH and TH frequency. Dots: experimental values. Line: fit obtained by the equation \( a \cdot I^n \) yielding \( n = 1.95 \) and \( 3.2 \) for SH and TH, respectively. B. Nonlinear axial PSF at SH and TH obtained with a 1.1 N.A. objective and 1250 nm excitation. Dots: experimental values. Line: Gaussian fit yielding FWHM=1.97 µm and 1.42 µm for SH and TH, respectively.

collection of multiple harmonics in nanometric systems with no phase-matching constraints can be very advantageous for increasing sensitivity in demanding applications, like ultra-sensitive detection in fluids as we recently demonstrated.\(^{19}\) For imaging, the use of > 1100 nm excitation wavelengths allowed by new laser sources ensures that standard microscope collection optics and acquisition detectors can be efficiently employed, moreover the two signals are very well spectrally separated and narrow which make their acquisition even easier.

To investigate the advantages of multi-harmonic detection in biomedically relevant samples, we proceeded in imaging excised cancer tissues from a xenograft tumor model. Details of preparation are provided in the S.I. Briefly, tumours were analysed that developed in female nude mice after implantation of human breast tumour cells MDA MB 231 either subcutaneously or orthotopically in the right abdominal mammary gland fat pad. Fresh breast tumor tissue sections were obtained using a vibratome, followed either by incubation with BFO HNPs or in buffer as control.

In Fig. 3 we show a multi-harmonic image of a unlabelled section from the breast orthotopic tumour (negative control). It is known that there exist strong endogenous sources of second (cyan, collagen)\(^{20}\) and third harmonic (yellow, lipids)\(^{21}\) in tissues, which are evident in the picture. Both these tissue constituents are abundant in tumours.\(^{22,24}\) Clearly, such harmonic background can affect the selective detection of HNPs. Some authors have already shown that HNPs can be imaged
by their SH emission against collagen background (mammalian tendon). However, it was also shown that for smaller HNPs the contrast was reduced, even though instrumental sensitivity is sufficient for detecting HNPs emission. Interestingly, the application of Costes’s algorithm to this image slice yields 0% co-localization events, as also visible in the scatter plot where elements along the diagonal are sorely missing.

The first column of Fig. 4 displays the SH signal of a tissue section from the subcutaneous breast tumour which was incubated with BFO HNPs. Collagen structures are clearly evident as diagonal stripes, the presence of small bright spots (sometimes at the limit of pixel resolution) is more pronounced than in the negative control of Fig. 3 and points to the presence of HNPs in the sample. Likewise, TH image in the central row shows the presence of small spots, together with other larger structures with different morphology with respect to collagen, which can be ascribed to lipids. The results of the co-localization procedure (SH∩TH, rightmost plot) enables highlighting in white exclusively pixels which show a simultaneous multi-harmonic emission and that, on the basis of the findings discussed in relation to Figs. 1 and 3, can be safely associated to the presence of BFO HNPs. The second row of Fig. 4 provides a more quantitative analysis of this co-localization effect. In general, the histograms of pixel intensity both at the SH and TH covers the whole detection dynamic range. Co-localization bars (in blue) indicate the number of occurrences which can be simultaneously attributed to both harmonic channels. One can see that the discrimination of HNPs signal against background emission of endogenous sources cannot
be based simply on intensity, as in both detection channels a relevant fraction of events at high intensity are not co-localizing.

![Figure 4: Representative multiphoton images of the subcutaneous breast tumour tissue labelled with BFO HNPs. First row: the first image (left square) shows the SH signal (cyan) in 400 um breast tumour section. The central image (middle square) shows the TH (yellow) signal at the same sample; and the third image (right square) the merged image with \( SH \cap TH \) colocalized pixels (white). Second row. SH and TH intensity histograms, blue bars correspond to the occurrence of co-localized events for the different intensity intervals. Scatter plot TH vs. SH. Blue points show events defined as co-localized by the algorithm.]

The application of multi-harmonic correlation for increasing selectivity by background rejection in optically congested environment like tissues can be performed in real-time, and therefore opens the way to HNPs tracking protocols\(^2^6\) with high selectivity and minimal image processing requirements. It is worth reminding that autofluorescence can be easily avoided when working in this wavelength range, as endogenous fluorophores two-photon absorption is prominent only \(< 800 \text{ nm}\)\(^2^7\). In addition, it is worth noting that resolution is generally not an issue for detecting HNPs. As demonstrated by the Beaurepaire group, it is advantageous when imaging thick samples by multiphoton excitation to employ objectives with low-magnification and large field of view / N.A.
to epi-collect efficiently multiple scattered photons.\textsuperscript{28}

In the light of all previous results, an interesting development for the use of HNPs for tissue imaging is their application as reference mapping structures for correlating light (multi-photon) and electron microscopy (CLEM), facilitating the retrieval of specific regions of interest going from one technique to the other.\textsuperscript{29} In this respect, BFO HNPs are particularly appealing, as they are electron dense and are expected to provide good contrast in electron-based imaging like TEM and SEM. Figure 5 shows HeLa-MZ cells labelled by BFO HNPs. Panel a shows a two-photon microscopy image of a single cell additionally labelled after fixation with Nile red (BFO NPs SH in cyan and fluorescence in red). SEM images of a different cell from the same culture are reported in panels b, c, and d. The difference among these images depends on the energy range of detected electrons and acceleration voltages. In fact, secondary electrons (SE), which are associated with 50 eV (or less) energy, provide mainly surface morphology information (Fig. 5 SE), while backscattered electron (BE, >50 eV) are sensitive to the composition of the specimen displaying brighter contrast for heavier elements (Fig. 5 BE). Overall, one can notice the good contrast provided by SEM for BFO HNPs. When detecting BE, by changing accelerating voltage one can modify the scattering region of the incident electron beam (see simulation in S.I.). Indeed in panel d, where the SEM accelerating voltage is set to 15 kV instead of 5 kV, one can see the appearance of HNPs below the cell surface, appearing as dimmer spots in the central region, likely coming from internalized BFO HNPs.\textsuperscript{7}

In conclusion, we have shown that multi-harmonic emission of BFO HNPs can be easily detected by multi-photon microscopy when using excitation >1100 nm. Very advantageously, the intensity difference among second and third nonlinear response is not large. Using the right combination of detectors for the different spectral ranges (GaAsP and standard photomultipliers) the two signals can be acquired simultaneously using standard settings. Based on this result, we have demonstrated that the co-localization of SH and TH allow identifying with unprecedented selectivity HNPs in a complex optical environment presenting endogenous sources of fluorescence and
harmonic generation, an excised xenograft tumour tissue in our experiment. The image processing necessary for this approach relies on simple two-channel co-localization, allowing its real-time use in demanding imaging applications. Moreover, by additional electron microscopy measurements, we have shown that BFO HNPs could prospectively serve as localization fiduciaries in advanced *in vivo* CLEM studies.\(^{30}\)

**Acknowledgement**

Authors are grateful to Cameron Christopher Scott for providing the MZ strain of HeLa cells and to Michel Moret for technical support. This research was partially supported by the European FP7 Research Project NAMDIATREAM (NMP4-LA-2010- 246479, [http://www.namdiatream.eu](http://www.namdiatream.eu)), Fondation pour la Recherche Médicale (FRM n. DGE20111123020), and the Cancéropôle Ile de France (n. 2012-2-EML-04-IC-1). The study was performed in the context of the European COST Action MP1302 Nanospectroscopy.
Supporting Information Available

Additional details on the experimental procedures. This material is available free of charge via the Internet at [http://pubs.acs.org/](http://pubs.acs.org/).

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