Sensitive Detection of Malaria Infection by Third Harmonic Generation Imaging

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ABSTRACT Malaria remains a major health concern worldwide, with 350–500 million cases reported annually in endemic countries. In this study, we report a novel and highly sensitive optical-based detection of malaria-infected blood cells by third harmonic generation (THG) imaging of hemozoin pigment that is naturally deposited by the parasite during its lifecycle. The THG signal from the hemozoin was greater than we have observed in any cell type with signal/noise ratios that reach 1000:1. This method allows a rapid and robust detection of early stage infections of blood cells. The immense nonlinear response of the intrinsic parasitic by-product pigments suggests that automated optical detection by THG could be used for sensitive and rapid screening of parasite infection in blood samples.

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Malaria remains a significant and increasing health and economic problem of profound dimensions. With the absence of efficacious new drugs in the pharmaceutical pipeline, a renewed effort to improve on the early detection and treatment of malaria is urgently needed. Moreover, such a highly sensitive method could prove to be a very reliable approach for screening of malaria-infected blood at blood banks. To improve the effectiveness of antimalarial treatments, there has been a shift from symptom-based to parasite-based diagnosis (1), especially for pregnant women and young children. This could be achieved using accurate and reliable detection methods with a low cost per test, especially in endemic parts of the developing world where the rates of malaria infections are high. One way to do this is to directly detect hemozoin (2), a digestion residue of the parasite, within infected red blood cells.

Currently, detection methods used in the field rely on DNA staining using Giemsa stain to label blood smears. The main advantages of DNA staining are its low cost (~5 cents per test) and the fact that an identification of the *Plasmodium* species(s) present in an individual is often possible. Although as low as 40 parasites per microliter of blood can be detected with Giemsa, this requires patience and skill of the microscopist/analyst; moreover, the quality of the diagnosis depends strongly on the skills and experience of a trained examiner (3) and it remains a time-consuming microscopy assay. As well, the process does not allow parallel multiple testing. Antigen detection, which requires minimal specific training, is the only method accessible for remote areas that can offer speciation of *Plasmodium* (i.e., *vivax* versus *falciparum*) and for which many tests can be performed simultaneously. However, the lowest number of parasites per microliter of blood that this rapid diagnostic test can detect is 400; in addition to having higher cost and poor quantification of parasitaemia (4). Furthermore, the high temperature and humidity in endemic regions accelerate the degradation of the reagents used for these rapid tests. Molecular techniques (such as polymerase chain reaction) have been suggested as an alternative to current detection methods (e.g., Giemsa staining); however, mutational changes to the diagnostic gene(s) or protein(s) often render such approaches less effective. Recently, malaria detection using a hematology analyzer was also achieved with promising results (5). Hence, despite advances in the diagnostic technologies available, misdiagnosis or overdiagnosis (and overtreatment) of malaria is still a common occurrence (6).

Third harmonic generation (THG) is a material-dependent nonlinear optical effect that involves the conversion of three photons of the fundamental laser frequency ω within the focus of a laser beam into one emitted photon of frequency 3ω due to the third-order dielectric susceptibility $\chi^{(3)}$ of the medium. Even for materials with a nonzero $\chi^{(3)}$, in bulk samples the destructive interference of photons generated on opposite sides of the beam focus leads to very low efficiency THG emission in the far field, so it has been restricted to imaging interfaces and small objects that are sites of inhomogeneities in $\chi^{(3)}$. Since its introduction in 1997 (7), THG microscopy has been used to image several different cell types, neurons (8), red blood cells (9), and also cellular lipid bodies (10) in live organisms. As with other variants of multiphoton microscopy, THG is a nonlinear process that provides inherent optical sectioning. It has been used to image unstained samples containing macromolecules or complexes with appropriate nonlinear optical properties (11). It is also possible to image at depth in scattering tissue, due to the

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deeper penetration of the infrared excitation laser wavelengths used for THG imaging.

Here we show that hemozoin produces a very strong THG signal at its interface and that this emission can be readily used to image malaria-infected red blood cells and to differentiate between infected and uninfected cells. Although hemoglobin in blood cells has been imaged by THG (9,12), and other porphyrin containing molecules are known to exhibit high $\chi^{(3)}$ due to large polarizable π-electron systems (13), hemozoin yields a THG signal that is up to three orders of magnitude larger that any other component found in our blood samples.

Images of hemozoin crystals in red blood cell samples infected with 3D7 and FCR-3 *P. falciparum* trophozoites were obtained using a home-built multiphoton microscope (see Supplementary Material). The images were collected in two detection channels via intrinsic two-photon absorption fluorescence (2PAF) of the red blood cells and THG from the hemozoin crystals, as is shown in Fig. 1 *a*. We confirmed a third order dependence of the emission on the excitation laser power and also used a spectrometer to verify that the emission frequency was the third harmonic of the laser fundamental frequency (see Supplementary Material).

To confirm that the third harmonic signal originated from within infected cells, blood smears were stained with Giemsa to label the DNA of the parasites and imaged using both bright field and THG microscopy. The bright field and THG images of the same regions within the samples showed perfect overlap for the locations of infected cells. The measured signal/noise (S/N) ratio of the THG generated in the hemozoin crystals reached 1000:1 (Fig. 1 *a*, *inset*), allowing a precise localization of the crystals in the images (Fig. 1, *a* and *c*). The measured THG signal from uninfected red blood cells was very weak and it was not possible to differentiate it from the background noise for the collection conditions used in these studies.

To further confirm that the THG emission was produced by malaria pigments, we imaged individual synthetic hemozoin crystals on glass coverslips as well as synthetic crystals being phagocytosed by macrophages (see Supplementary Material).

We also verified that THG detection could be used at different life cycle stages and with different strains of parasites, by performing a comparative imaging study of the parasitaemia level of synchronized parasites from two different strains using Giemsa and THG detection methods. The difference in the parasitaemia level obtained with the two approaches was $<1\%$ except for the 3D7 ring stage sample (see Supplementary Material). It has to be noted that hemozoin from the ring stage parasites produces a dimmer signal than those imaged for the trophozoite stage as there is less hemozoin present (Fig. 1, *e–h*); however, the S/N still ranged between 30 and 100 with a mean of 62. The slightly higher parasitaemia level obtained from the THG image particle counts can be explained by the fact that free hemozoin bound to the surface of an uninfected cell can be mistaken for an infected cell by the examiner. However, the difference in measured levels was still $<2\%$ for the 3D7 ring stage sample.

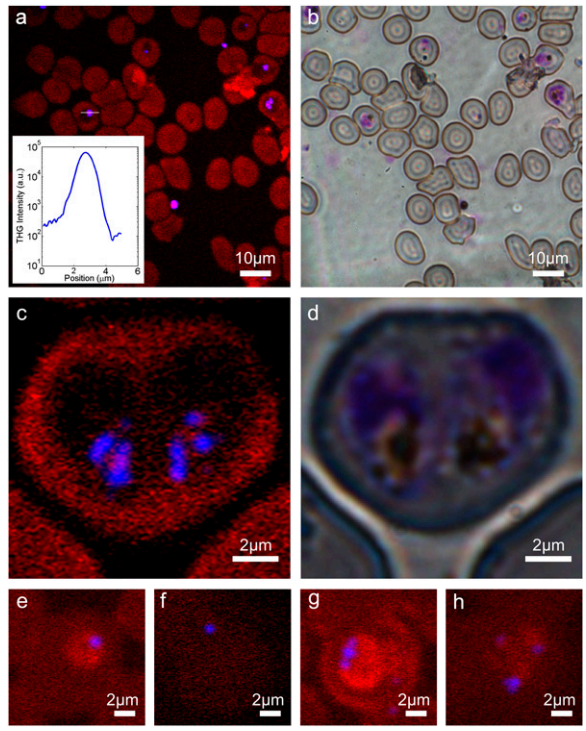

FIGURE 1 Multiphoton imaging of Hemozoin. THG signal is localized to infected blood cells as verified by imaging of standard Giemsa-stained thin blood films. (*a* and *b*) 3D7 *P. falciparum* infected red blood cells from Giemsa-stained thin blood smears imaged by multiphoton (*a*) and bright field light microscopy (*b*). The multiphoton image (*a*) consists of superimposed THG (*blue*) from hemozoin and intrinsic 2PAF (*red*) from red blood cells. The 2PAF channel image was the average of 20 sequential frame scans whereas the THG channel image was acquired in only one scan frame with a beam dwell time of 5 μs per pixel. The inset shows the THG intensity lineout plot in logarithmic scale for the hemozoin crystal bisected with a line in the image (*a*). The S/N for this particular crystal is slightly >1000. The locations of the hemozoin in *a* and *c* correlates with the dark spots in *b* and *d*. (*c* and *d*) We could readily resolve cells with a double infection by *P. falciparum* as can be seen from the THG and 2PAF signal (*c*) and its overlap with the bright field imaged dark spots of the hemozoin crystals shown in *d*. We also show typical single scan images of infected blood cells taken from synchronized strain: 3D7 ring (*e*), 3D7 ring and early trophozoite (*f*), FCR-3 trophozoite (*g*), and FCR-3 ring and early trophozoite (*h*).

Two aspects of the molecular structure and composition of hemozoin can explain the high third order optical susceptibility of malaria pigments. Hemozoin is a condensed phase of iron(III)porphyrin rings, which form an extensive polarizable π-electron system (2). Secondly, considering the optical excitation parameters used in our microscope, the optimal size for the THG chromophore should be close to the illumination wavelength. The needle-shaped malaria pigments have dimensions ranging from 0.2 to 1.0 μm, which match the theoretical optimum for imaging by THG using the collection conditions employed.

Automated malaria detection by flow cytometry in combination with fluorescence staining was previously investigated (14), but ''background noise'' limited the detection to >2000 par/μL of blood. However, in comparison, our THG images have much higher S/N ratios for an integration time of 5 μs per pixel. Such high signal levels should permit flow cytometry with THG based detection. Considering these parameters (which set limits for the flow speed), the focal volume, the average hematocrit level, and the dimensions of the red blood cells, we estimate that to detect 40 par/μL in 50 μL of blood by detecting a total of 10 infected cells would take on average 5.5 min. Our estimate is based on our imaging setup, but a higher laser power and lower numerical aperture objective could easily increase the rapidity of such a device by one order of magnitude. We envisage a turn-key THG/flow cytometer system based around a short pulse fiber laser operating at a fixed wavelength in the infrared that would provide automated counts of infected cells. This system would require only blood sample injection, but without the need for staining, and could therefore be operated by personnel with minimal training.

We have established that malaria parasite infections can be specifically detected in infected red blood cells by imaging THG emission from the hemozoin using infrared femtosecond pulsed laser excitation. The combination of the very high S/N ratios obtained the intrinsic digestive by-product of the parasite and the lack of other comparable sources of THG in the blood suggests that our approach can be used as a very sensitive indicator of malaria infection. Existing technology suggests that a flow cytometry device could be adapted using THG emission for automated stain-free diagnosis based on parasitaemia counts, which would also lower the minimum parasitaemia levels that are detectable. This will be beneficial for many countries, some of which test ~100 million blood samples every year. Furthermore, we expect it will help lower the financial barrier to permit systematic testing in endemic areas.

SUPPLEMENTARY MATERIAL

To view all of the supplemental files associated with this article, visit www.biophysj.org.

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REFERENCES and FOOTNOTES

1. Bell, D., C. Wongsrichanalai, and J. W. Barnwell. 2006. Ensuring quality and access for malaria diagnosis: how can it be achieved? *Nat. Rev. Microbiol.* 4:682–695.
2. Pagola, S., P. W. Stephens, D. S. Bohle, A. D. Kosar, and S. K. Madsen. 2000. The structure of malaria pigment beta-haematin. *Nature*. 404: 307–310.
3. Lema, O. E., J. Y. Carter, N. Nagelkerke, M. W. Wangai, P. Kitenge, S. M. Gikunda, P. A. Arube, C. G. Munafu, S. F. Materu, C. A. Adhiambo, and H. K. Mukunza. 1999. Comparison of five methods of malaria detection in the outpatient setting. *Am. J. Trop. Med. Hyg.* 60: 177–182.
4. Roll Back Malaria (RBM) Partnership. 2004. Sources and Prices of Selected Products for the Prevention, Diagnosis and Treatment of Malaria. RBM Partnership, Geneva.
5. Fawzi, Z. O., N. A. Fakhro, R. A. Nabhan, A. Alloueche, and C. S. Scott. 2003. Differences in automated depolarization patterns of *Plasmodium falciparum* and *P. vivax* malaria infections defined by the Cell-Dyn CD4000 haematology analyser. *Trans. R. Soc. Trop. Med. Hyg.* 97:71–79.
6. Hamer, D. H., M. Ndhlovu, D. Zurovac, M. Fox, K. Yeboah-Antwi, P. Chanda, N. Sipilinyambe, J. L. Simon, and R. W. Snow. 2007. Improved diagnostic testing and malaria treatment practices in Zambia. *JAMA*. 297:2227–2231.
7. Barad, Y., H. Eisenberg, M. Horowitz, and Y. Silberberg. 1997. Nonlinear scanning laser microscopy by third harmonic generation. *Appl. Phys. Lett.* 70:922–924.
8. Yelin, D., and Y. Silberberg. 1999. Laser scanning third-harmonic-generation microscopy in biology. *Opt. Express*. 5:169–175.
9. Millard, A. C., P. W. Wiseman, D. N. Fittinghoff, K. R. Wilson, J. A. Squier, and M. Muller. 1999. Third-harmonic generation microscopy by use of a compact, femtosecond fiber laser source. *Appl. Opt.* 38:7393–7397.
10. Debarre, D., W. Supatto, A. M. Pena, A. Fabre, T. Tordjmann, L. Combettes, M. C. Schanne-Klein, and E. Beaurepaire. 2006. Imaging lipid bodies in cells and tissues using third-harmonic generation microscopy. *Nat. Methods.* 3:47–53.
11. Zipfel, W. R., R. M. Williams, and W. W. Webb. 2003. Nonlinear magic: multiphoton microscopy in the biosciences. *Nat. Biotechnol.* 21:1369–1377.
12. Clay, G. O., A. C. Millard, C. B. Schaffer, J. Aus-der-Au, P. S. Tsai, J. A. Squier, and D. Kleinfeld. 2006. Spectroscopy of third-harmonic generation: evidence for resonances in model compounds and ligated hemoglobin. *J. Opt. Soc. Am. B*. 23:932–950.
13. Anderson, H. L., S. J. Martin, and D. D. C. Bradley. 1994. Synthesis and third-order nonlinear optical properties of a conjugated porphyrin polymer. *Angew. Chem. Int. Ed. Engl.* 33:655–657.
14. Janse, C. J., and P. H. Van Vianen. 1994. Flow cytometry in malaria detection. *Methods Cell. Biol.* 42 Pt. B:295–318.