Rotating-crystal Malaria Diagnosis: Pre-clinical validation

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Improving the efficiency of malaria diagnosis is one of the main goals of current malaria research. We have recently developed a magneto-optical (MO) method which allows high-sensitivity detection of malaria pigment (hemozoin) crystals via their magnetically induced rotation in blood. Here, we validate this technique on laboratory derived blood samples infected with Plasmodium falciparum. Using two parasite cultures, the first containing mostly ring stages and the second corresponding to the end of the parasite life cycle, we demonstrate that our novel method can detect parasite densities as low as ~40 and ~10 parasites per microliter of blood for ring and schizont stage parasites, respectively. This detection limit exceeds the performance of rapid diagnostic tests and competes with the threshold achievable by light microscopic observation of blood smears. Our method can be performed with as little as 50 microliter of capillary blood and is sensitive to the presence of hemozoin micro-crystals down to ppm concentrations. The device, designed to a portable format for clinical and in-field tests, requires no special training of the operator or specific reagents, except for an inexpensive lysis solution to release intracellular hemozoin. Beyond diagnostics, this technique may offer an efficient tool to study hemozoin formation, trace hemozoin kinetics in the body and test susceptibility/resistance of parasites to new antimalarial drugs inhibiting hemozoin formation.

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Introduction

Although there is a plethora of emerging techniques aiming at high-sensitivity diagnosis of malaria, only a few of these approaches are feasible for clinical and in-field diagnosis. Apart from purely symptom based, presumptive diagnosis, the two main diagnostic methods currently in practice are the antigen-based detection of malaria parasites using rapid diagnostic tests (RDT) and the microscopic observation of infected red blood cells in blood smears.1–4 The detection limits of RDT and light microscopy have been reported to be approximately 100 parasites/µL and 5–50 parasites/µL, respectively.5–7 Both of these methods are subject to inherent limitations: i) although RDTs are becoming more affordable, they cannot provide a quantitative measure of parasitemia and presently do not possess sufficient sensitivity to detect low-level infections which are very common in endemic settings, ii) the visual inspection of blood smears is time and labor intensive. Moreover, the detection threshold of 5 parasites/µL is rather theoretical and can only be achieved under ideal conditions (good-quality blood film, highly trained microscopist, high-powered microscope, etc.). In practice, most routine diagnostic laboratories achieve approximately 50 parasites/µL and detect about 50% of malaria cases.6,8–9

Among molecular biology-based methods, polymerase chain reaction (PCR) assays surpass the performance of RTDs and light microscopy.10,11 However, they often require expensive equipment and reagents, highly trained laboratory personnel and are prone to contamination.12 Recent studies conclude that real-time PCR has a detection limit corresponding to a few parasites in 1 µL blood,13,14 nevertheless, it is not yet a practical method for routine diagnosis under field conditions.

The idea to take advantage of the unique magnetic properties of malaria pigment (hemozoin) and to use it as an alternative target of optical diagnosis has been proposed by several groups.15–19 Hemozoin is a micro-crystalline heme compound produced by malaria parasites as they detoxify free heme derived from hemoglobin digestion. Our recent study using synthetic hemozoin crystals suspended in blood demonstrated that the rotating-crystal magneto-optical (MO) diagnostic method can detect hemozoin concentrations down to 15 pg/µL.20 This threshold concentration was estimated to be equivalent to a parasite density of ≤30 parasites/µL in infected blood provided that the whole amount of hemozoin produced by the parasites is released into the lysed cell suspension. However, the relation between hemozoin concentration and parasite density in human infections is not straightforward. Intraerythrocytic hemozoin content is dependent on the maturity of the blood stage parasites; with the least amount of hemozoin present in erythrocytes during the ring stage and the highest amount during...
the schizont stage 20–22. Therefore, the hemozoin concentration in blood derived from human infections depends on the parasite stage distribution at the time the blood sample was collected. In *Plasmodium falciparum* (the most lethal parasite species) infections often only the ring and early trophozoite stages are found in the peripheral circulation, since the later developmental stages cytoadhere to the vascular endothelium 23.

Moreover, the MO signal recorded by our technique depends not only on the amount but also on the size and morphology of the hemozoin crystals, which can be different for synthetic and naturally grown crystals 24–26. The MO method is sensitive only to those hemozoin crystals released into suspension, which can be magnetically rotated. Correspondingly, aggregation of crystals or their binding to other components of lysed infected blood, such as cell membranes, could substantially decrease the sensitivity of our technique. Signal loss may be avoided by appropriate lysis and treatment of blood samples prior to measurement.

In the present study we aimed to address these key issues and to validate the rotating-crystal malaria diagnosis method using synchronized cultures of *P. falciparum*. (For a short description of the detection scheme see Materials and Methods section.) For this purpose, we investigated its sensitivity and detection threshold for two cultures with different maturity distributions of the parasites. The first, hereafter referred to as the *ring stage culture*, contained mostly ring stages and some early trophozoites with a total parasite density of P≈3.1 × 10⁶ parasites/µL. It is representative to the distribution of parasite blood stages most often encountered in *P. falciparum* infections. The second with a lower total parasite density of P≈2.8 × 10⁴ parasites/µL corresponds to the end of the parasite life cycle where some of the parasites are still in the schizont form but most of them, following an invasion, already turned to early-stage rings of the next generation. The distributions of the parasites among the different stages – early-ring, late-ring, early-trophozoite, late-trophozoite, early-schizont and late-schizont stages – in the two cultures are displayed in Fig. 1 together with light microscope images of parasitized thin blood films containing infected red blood cells.

**Results**

The MO signal, the measure of hemozoin content within the lysed cell suspension, is shown in Fig. 2 for dilution series of the ring and schizont stage cultures. The 20 serial 2-fold dilutions using uninfected erythrocytes allowed for MO signal to be assessed over 6 orders of magnitude of parasitemia. As a general trend in Fig. 2A, the MO signal varies proportionally to the parasitemia level...
and the signal for each sample shows a gradual decrease with increasing frequencies of the rotating magnetic field. This frequency dependence is in agreement with previous results obtained when using synthetic hemozoin crystals suspended in blood and originates from the viscosity of the lysed cell suspension hindering fast rotations of the crystals.

As schizont stages contain more hemozoin than ring stage-parasites, samples from the diluted schizont stage culture exhibited higher signals than ring stage samples with comparable parasitemia. Although the overall frequency dependence is similar for the two cultures, the decrease in the signal with increasing frequency is more pronounced for schizont stage samples, which is likely due to the larger crystal size in these samples. At low levels of parasite density, namely for samples with $P≤10$ parasites/μL from the ring stage culture, the signal does not further drop with decreasing parasitemia. The frequency dependence of the MO signal for these ring stage samples also becomes different; the low-frequency saturation common for higher concentrations does not hold anymore. These imply a residual MO signal not related to hemozoin.

In order to determine the detection limit of our method, the results obtained for the dilution series of the ring and schizont stage cultures are summarized in Fig. 2B, where the MO signal at 20 Hz is plotted versus the parasitemia (and parasite density). Sequential measurements performed on the same sample with time delays less than one hour gave identical results. In several cases we checked the reproducibility of the protocol by repeating the measurement for both of the duplicate sam-

FIG. 2: Magneto-optical (MO) detection of parasitemia in synchronized *Plasmodium falciparum* cultures. Panel A: Red and blue curves show the frequency dependent MO signal for samples from the ring and schizont stage cultures, respectively, with various levels of parasite density given in μL$^{-1}$ units on the right of the respective curves. For ring stage samples with parasite densities lower than 10 parasites/μL, the MO signal does not further decrease. Data plotted with triangles and diamonds are the residual signal from freshly hemolyzed uninfected blood and water, respectively. The frequency scale denotes twice the rotation frequency of the magnetic field, i.e. the frequency of the modulated intensity. Panel B: Red and blue curves show the frequency dependent MO signal for samples from the ring and schizont stage cultures, respectively. Solid and open squares correspond to the duplicate samples labeled as samples #1 and samples #2. Triangles indicate the results obtained by remeasuring samples #1 with 24h delay. The solid lines following the trend of the MO signal at higher parasite densities for ring (red line) and schizont (blue line) samples are guides for the eye. The black horizontal line represents the mean residual MO signal for the cultures, while the 95% confidence levels of this mean detection limit for the ring and schizont stage samples are indicated by red and blue dashed lines, respectively. Correspondingly, for ring and schizont stage samples with parasite density higher than 40 parasites/μL and 10 parasites/μL, respectively, the diagnosis is positive with a confidence of at least 95%. The background signal for freshly hemolyzed uninfected blood and water are also shown by dark and light grey lines. All these horizontal indicators are also shown in panel A for reference. The upper horizontal scale shows the corresponding levels of parasitemia.
amples labeled as samples #1 and samples #2 in Fig. 2B. For ring stage samples with \( P \leq 10 \) parasites/\( \mu L \), where the MO signal shows no systematic variation with decreasing parasite density, we calculated the mean value of the residual MO signal \((\approx 3.6 \times 10^{-4} \%)\) and its standard deviation \((\approx 1.5 \times 10^{-4} \%)\). Assuming Gaussian distribution for the residual MO signal values, we found that the 95% confidence level of the mean detection limit for ring stage samples is \( DT/T = 6.6 \times 10^{-4} \%), which corresponds to a parasite density of \( \sim 40 \) parasites/\( \mu L \). This is equivalent to the parasitemia level of \( 8 \times 10^{-4} \%). Since the reproducibility is poorer for schizont stage samples, in this case our rough estimate for the 95% confidence level of the mean detection limit is considerably higher with \( DT/T = 2 \times 10^{-3} \%)\), which corresponds to the parasite density of approximately \( \sim 10 \) parasites/\( \mu L \) and a parasitemia level of \( 2 \times 10^{-4} \%). Note that the reproducibility between duplicate samples does not significantly vary with parasite density for dilutions of either the ring or the schizont stage cultures.

The hemozoin content of the two cultures can be roughly estimated from the parasite density and the stages of parasite development specified in Fig. 1. Ring and early trophozoite stages up to 24 h were reported to convert about 3-15% of the total hemoglobin in the infected red blood cells to hemozoin, while at the schizont stage this portion is increased to approximately 50-80%. According to the 3-15% hemoglobin conversion rate reported for rings and early trophozoites, the undiluted ring stage culture with total parasite density of \( P = 3.1 \times 10^4 \) parasites/\( \mu L \) contained \( \sim 9-48 \) ng/\( \mu L \) hemozoin. The undiluted schizont stage culture, which had about 10 times lower parasite density with \( P = 2.8 \times 10^4 \) parasites/\( \mu L \), contained all the hemozoin formed during the first cycle. Using the hemoglobin conversion rates quoted above, this corresponds to \( \sim 14-23 \) ng/\( \mu L \) hemozoin. An independent estimate, based on the MO signal yields approximately \( 6 \) ng/\( \mu L \) and \( 9 \) ng/\( \mu L \) for the undiluted ring and schizont stage cultures, respectively. For this estimate, we used the conversion factor \( c_{Hz} = 1 \) ng/\( \mu L \) \( \rightarrow DT/T = 1.4 \% \) between the hemoglobin concentration and the low-frequency \((\sim 1 \text{ Hz})\) MO signal previously determined for artificial hemozoin crystals suspended in blood. Note that the 20-fold dilution of the samples prior to the MO measurement needs to be taken into account, since this conversion factor applies for samples with 50% hematoctrit.

We also estimate the hemozoin concentration of the two undiluted cultures based on MO signal using the conversion factor \( c_{Hz} = 1 \) ng/\( \mu L \) \( \rightarrow DT/T = 1.4 \% \) between the hemozoin concentration and the low-frequency \((\sim 1 \text{ Hz})\) MO signal previously determined for artificial hemozoin crystals suspended in blood. This yields approximately \( 6 \) ng/\( \mu L \) and \( 9 \) ng/\( \mu L \) for the undiluted ring and schizont stage cultures, respectively. Note that the 20-fold dilution of the samples prior to the MO measurement needs to be taken into account, since this conversion factor applies for samples with 50% hematoctrit.

The first scenario is supported by the electron microscopy images in Fig. 3, where hemozoin crystals inside of infected erythrocytes and as extracted from the cultures are shown. These elongated crystallites are considerably smaller (with typical length of \( \sim 200-500 \) nm) than the synthetic ones \((\sim 500-900 \) nm) previously studied and also displayed in the figure. This is also in accordance with the weaker frequency dependence of the MO signal observed in the present study, which indicates that natural crystals are able to follow the rotation of the magnetic field up to higher frequencies than the synthetic ones as a possible consequence of their reduced size. Furthermore, the comparison between the natural crystals within the parasites and those extracted from the cultures confirms...
no major change either in the size or in the morphology of the crystals due to our lysis-sonication protocol.

In order to minimize binding and aggregation of crystals we repeated the measurements 24 h later following another 30 min of sonication. We found modest but systematic increase of the MO signal of typically not more than 10-30%. On this basis, we expect that most of the hemozoin is successfully released into suspension, likely in the form of individual crystals.

The presence of a frequency-dependent residual MO signal indicates that some components of lysed blood can be magnetically oriented and rotated similarly to the hemozoin crystals. We have also studied freshly drawn blood following the same lysis protocol. The residual MO signal observed in this case was considerably lower than found for either the ring or schizont stage samples (see Fig. 2), which implies better detection threshold for instant diagnosis. We suspect that due to the freeze-thaw-lysis procedure applied to the present set of samples, some portion of the hemoglobin may have been transformed to an aggregated or polymerized form – similar to the intracellular non-covalent polymerization of hemoglobin previously observed in sickle cell disease, which may produce the residual MO signal. These points, requiring additional systematic studies, stress the crucial role of an appropriate blood treatment prior to diagnosis.

The noise floor of our equipment, determined using pure water, is roughly frequency independent and about one order of magnitude smaller than the residual signal from fresh blood. This enables further improvement of the detection limit provided that the residual MO signal from blood can be reduced by optimizing blood treatment. Optimizing the properties of the lysis solution may also help to dissociate the crystals without the need for sonication.

**Discussion**

The potential of exploiting hemozoin as a magnetic biomarker for malaria diagnosis has stimulated extended research over the last few decades. Taking advantage of the paramagnetic nature of hemozoin, several approaches have been proposed to improve the sensitivity of existing methods by the magnetic separation of malaria infected erythrocytes from whole blood prior to the diagnosis. More recently, new techniques have been emerging, which directly use hemozoin as a target material of magnetic diagnosis. These techniques include detection of depolarized side-scatter in flow cytometry, electrochemical magneto immunoassays, magnetically enriched surface enhanced resonance Raman spectroscopy and magneto-optical detection using polarized light. Among them, to the best of our knowledge, our rotating crystal MO diagnostic device is the first realized in a cost-effective portable format with excellent sensitivity.

In the present study, the detection limit of our rotating-crystal MO diagnostic device was found to be ~40 parasites/µL and ~10 parasites/µL for ring and schizont stage parasites, respectively. These detection limits are below the threshold currently achievable with RDTs (>100 parasites/µL) and lie within the same range as the limits of conventional optical microscopy for malaria diagnosis (5-50 parasites/µL). For the present set of blood samples kept frozen and thawed before the measurement, the performance of the method was limited by a residual MO signal due to some part of the lysed cell suspension. This residual MO signal obscures the genuine MO signal of hemozoin at parasite densities lower than the limits quoted above. Preliminary results indicate that for measurements on freshly lysed blood samples, which is the condition relevant to instant diagnosis, the detection limit of our rotating crystal MO platform could be further improved. Thus, this methodology has the potential to yield portable tools for instant diagnosis with detection limits approaching that of PCR-based platforms.

Limitations of our diagnostic technique include i) the possibility of false positive detections due to the presence of hemozoin in the blood, e.g. contained within white blood cells, for extended periods of time after an infection has been cleared and ii) the possibility of false negative results in case an infection only contains very early ring stage parasites with little or no hemozoin. Furthermore, methods targeting only hemozoin as a marker for infection are thought to have limitations in their diagnostic capacity as they cannot distinguish between different malaria species. The specificity of our MO diagnostic scheme, owing to variations in the typical size and morphology of hemozoin crystals produced by different species, needs to be tested by a comparative study on various Plasmodium strains. We emphasize that only studies on field isolates will be able to elucidate the impact of these possible confounding factors and the present study is the basis for such field-based trials.

It is currently believed that without active case detection of asymptomatic malaria infections, malaria eradication will be impossible or very difficult to achieve. However, there are no diagnostic tools for rapidly screening hundreds of people per day, on-site and with high sensitivity. The rotating-crystal MO diagnostic device has the potential to fulfill these requirements as it is cost-effective, rapid, highly sensitive, portable and easy to apply.

Besides on-site diagnosis, the present methodology provides an efficient in-vitro laboratory tool to test the susceptibility of the parasites to new antimalarial drugs by monitoring the effect of treatment on the rate of hemozoin formation. The scope of the technique described here may also cover the study or diagnosis of other human diseases, such as schistosomiasis, which are also caused by blood-feeding organisms producing hemozoin similarly to malaria parasites.
Materials and Methods

Parasite culture

P. falciparum parasites (laboratory adapted strain 3D7) were cultured following the method of Trager and Jensen with modifications. The culture medium was RPMI 1640 with L-glutamine (GIBCO cat # 31800) supplemented with 2 mg/mL NaHCO3 (Merck, cat # 106329), 25 mg/L gentamicin (Pfizer, cat # 61022027), 50 mg/L hypoxanthine (Calbiochem, cat # 4010), 25 mM HEPES (SAFC, cat # 90909C) and 10% pooled O+ human serum (mixed blood groups, Australian Red Cross Blood Service). Cultures were maintained at 4% hematocrit with changes of culture medium every 48 h and diluted with uninfected O+ red blood cells when the parasitemia exceeded 5%. Parasites were maintained in an atmosphere of 5% CO2 and 1% O2 in N2. Parasite cultures were kept in stage synchrony by applying the 5% Sorbitol method, first described by Lambros and Vanderberg. Hemozoin liberated from late stage parasites by the synchronization process was removed by washing the cells in RPMI medium after the Sorbitol induced cell lysis and before re-establishment of the culture.

Parasite densities for the the ring and schizont stage cultures described above were estimated by counting the number of parasites contained in 5000 red blood cells on Giemsa stained thin blood films. The commonly used conversion from parasitemia to parasite density based on the assumption that 1 ml of blood contains 5 x 109 red blood cells at 50% hematocrit was applied. For both, the ring and schizont stage cultures, 2-fold dilution series were prepared in duplicate in uninfected human erythrocytes (Red Cross blood bank, Royal Melbourne Hospital, VIC, Australia). The duplicate dilution series prepared for ring and schizont cultures contained 21 and 20 dilutions, respectively, where each of the dilutions had a volume of 200 µL at a hematocrit of 50%. These dilutions were immediately frozen and thawed twice to create lysates. These lysates, hereafter referred to as ring and schizont stage samples, were subsequently frozen at -80°C and kept in a frozen state until they were thawed immediately prior to measurement. The personnel carrying out the MO measurements were blinded against the contents of each of the samples to reduce potential observer bias.

Blood treatment prior MO diagnosis

Blood samples prepared for MO measurement were thawed at room temperature and were diluted 20-fold with distilled water, resulting in a total volume of 4 mL per specimen. Additionally, 100 µL of a special red cell lysis buffer, hereafter referred to as clearing solution, was added to the specimens in order to disperse the remnants of the lysed RBCs. In preliminary experiments, this clearing solution (2.5 V/V% Triton X-100 in 0.1 M NaOH) was confirmed not to cause noticeable degradation of hemozoin when added to synthetic malaria pigment suspended in lysed blood. Note that the final concentration of NaOH in the samples was only 2.5 mM. The samples were subjected to 30 min of ultrasonication to dissociate potential aggregates of hemozoin and ensure an unhindered motion of crystals in the fluid. Owing to this treatment a transmittance of ~30-45% could be achieved with no substantial light scattering observed from the blood samples. MO signals were recorded on 1 mL volumes taken from the samples subsequent to the preparation process and reproducibility was confirmed in several cases after one day of storage of the samples at 4°C and by carrying out the measurement on both of the duplicate samples.

Magneto-optical measurements

MO measurements were performed with the prototype of the rotating magnet setup described in our previous study. We utilize a permanent magnetic ring, which produces a B=1 T magnetic field at the sample position and can be rotated with adjustable frequency. Polarized light from a laser diode is transmitted through the sample in the direction perpendicular to the plane of the rotating magnetic field. Owing to the magnetic alignment of the freely rotating and dichroic hemozoin crystals present in infected blood, magnetically induced linear dichroism can be observed and quantified as the difference in transmission for light polarized along and perpendicular to the magnetic field direction (ΔT) divided by the average transmission (T), i.e. ΔT/T. Due to the synchronous periodic rotation of the crystals the linear dichroism gives rise to a periodic change in the transmitted intensity oscillating with twice the magnet rotation frequency. This second harmonic intensity component (I2f) can be effectively filtered by a lock-in technique. The MO signal is then obtained as the ratio of the modulated light intensity and the average intensity (Iav), i.e. ΔT/T = I2f/Iav.

This detection scheme for malaria diagnosis provides the best signal-to-noise ratio in the rotation frequency regime of 10-30 Hz. In order to exclude baseline artifacts emerging from mechanical vibration or improper optical alignment of the system, the baseline is checked with pure distilled water prior to the measurement of each sample. This water baseline – due to electronic and mechanical noise – was generally found to be almost two orders of magnitude lower than the signal from the samples with the lowest hemozoin concentration.

Electron microscopy

For transmission electron microscopy (TEM), parasite samples were fixed in resin blocks and 70-120 nm thin sections were cut using a Leica EM UC6 microscope (Leica Microsystems, North Ryde, NSW, Aus-
tralia) and brought onto carbon coated copper TEM grids (ProSciTech, Thuringowa, Qld., Australia). The TEM grids were then stained with 5% uranyl acetate for 15 min and Reynold’s lead citrate solution for 5 min. TEM was conducted on a JEOL 2100 TEM (JEOL Inc., Tokyo, Japan). For details of the method see the Supporting Information.

For scanning electron microscopy (SEM), the samples giving the highest MO signal were used and hemozoin crystals were extracted following the method of Chen and coworkers. The dark brown pellet obtained by this method was resuspended in 80 µL water. For SEM imaging small droplets of the suspension containing the hemozoin crystals were applied to gold coated glass slides without further purification or treatment. The droplets were dried overnight at room temperature. The SEM images were acquired on a LEO 1540XB electron microscope using the in-lens detector. The accelerating voltage was set to 3 kV and the viewing angle was perpendicular to the gold surface.

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