Micro Magnetic Resonance Relaxometry for label-free, rapid malaria diagnosis

Weng Kung Peng^{1*&}, Tian Fook Kong^{1,2*}, Chee Sheng Ng^{3,4*}, Lan Chen¹, Yongxue Huang¹, Ali Asgar S. Bhagat¹, Nam-Trung Nguyen^{1,2#}, Peter Rainer Preiser^{3,4&}, Jongyoon Han^{1,5,6&}

¹BioSystems & Micromechanics IRG (BioSyM), Singapore-MIT Alliance for Research and Technology (SMART) Centre, 1 Create Way, #04-13 Enterprise Wing, Singapore 138602

²School of Mechanical and Aerospace Engineering,
Nanyang Technological University, 50 Nanyang Avenue, Singapore.

³School of Biological Sciences,
Nanyang Technological University, 60 Nanyang Drive, Singapore.

⁴Infectious Diseases IRG (ID), Singapore-MIT Alliance for Research and Technology (SMART) Centre, 1 Create Way, #03 Enterprise Wing, Singapore 138602

⁵Department of Electrical Engineering and Computer Science, Massachusetts Institute of Technology, 36-841, 77 Massachusetts Avenue, Cambridge, MA 02139

⁶Department of Biological Engineering, Massachusetts Institute of Technology, 36-841, 77 Massachusetts Avenue, Cambridge, MA 02139

We report a new technique for sensitive, quantitative and rapid detection of *Plasmodium spp.* infected red blood cells (iRBCs), by means of Magnetic Resonance Relaxometry (MRR). During the intra-erythrocytic cycle, malaria parasites metabolize large amounts of cellular hemoglobin and convert it into hemozoin crystallites. We exploit the relatively large paramagnetic susceptibility of these hemozoin particles, which induce substantial changes in the transverse relaxation rate of proton nuclear magnetic resonance of RBCs to infer the 'parasite load' in blood. Using an inexpensive bench-top 0.5 Tesla MRR system, we show that with minimal sample preparatory steps and without any chemical or immuno-labeling, a parasitemia level of less than 10 parasites μL^{-1} in a volume below 10 μL of whole blood is detected in a few minutes. This method is both demonstrated for cultured *Plasmodium falciparum* parasites and *in vivo*, with *Plasmodium berghei* infected mice.

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Keywords:

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*Authors have equal contributions. &Corresponding Authors: Jongyoon Han(jjhan@mit.edu, Tel: +1 617 253 2290), Weng Kung Peng(wengkung@smart.mit.edu, Tel: +65 6516 1349), Peter Rainer Preiser(ppreiser@ntu.edu.sg, +65 6316 2822). #Current address: Queensland Micro- and Nanotechnology Centre, Griffith University, Brisbane, Queensland 4111, Australia.

INTRODUCTION

Malaria is one of the most lethal infectious diseases in tropical regions. Among the five protozoan species of *Plasmodium* that infect humans, *P. falciparum* parasites cause the highest and most severe disease burden, accounting for 90% of total deaths. The current 'gold-standard' for malaria diagnosis is the microscopic examination of Giemsa-stained blood smears¹. Depending on experience and the availability of fresh reagents a microscopist may be able to identify as little as 0.001% parasitemia from a thick-film blood smear^{2,3}. Thin blood smears provide much clearer images but suffer from reduced sensitivity^{4,5}, while polymerase chain reaction (PCR) based diagnostic approaches are often not practical for on-site application⁴. In addition, both PCR and Rapid Diagnostic Tests (lateral-flow immunoassay of parasite-specific proteins) are unable to provide quantitative analysis on the parasitemia level^{2,4} (Supplementary Table 1).

In this work, we report a bench-top Magnetic Resonance Relaxometry (MRR) system which is capable of rapid detection and quantification of the parasite load during the early stage of infection^{6,7} (Fig. 1). Traditional equipment for Nuclear Magnetic Resonance (NMR) spectroscopy is expensive and bulky, thus difficult to be adapted as an on-site diagnostic tool. Recently, however, Weissleder and coworkers scaled down the typical NMR system for point-of-care medical diagnostics^{8,9}. Based on this, we have developed a low-cost, portable field-programmable gate array based system and demonstrated that one can indirectly deduce the relative bulk magnetic susceptibility of the blood cells within a few minutes of signal acquisition time^{10,11}, without expensive and extensive immuno-labeling procedures.

In this work, we exploit the presence of the hemozoin crystallites formed within the erythrocyte as early as in the ring stage^{12,13} as a natural magnetic label for MRR detection of *Plasmodium spp* infected red blood cells (iRBCs) (Fig. 1c). During the intra-erythrocytic cycle, malaria parasites consume hemoglobin and release free heme. Free heme is immediately converted into an insoluble crystallite known as hemozoin. The formation of hemozoin represent a simple transformation in the redox state of the RBCs; from ferrous, Fe^{2+} (diamagnetic state) into ferric, Fe^{3+} (paramagnetic state), which causes a major change in bulk magnetic susceptibility of the iRBCs^{14,15}, and induces measurable changes in the magnetic resonance relaxation of nearby protons^{16,17}.

With an inexpensive portable bench-top system and minimal sample preparation, we demonstrate that, the system is able to detect *P. falciparum* infection to as low as 0.0002% parasitemia ($P < 0.0003$) using 750 nL of cultured blood, in less than 5 min. We further demonstrate using an *in vivo* *P. berghei* mouse infection model a strong correlation ($R^2 > 0.96$)

between R_2 -index measured by MRR and blood smear microscopy, allowing reliable estimation of parasitaemia to as low as 0.0001% (< 10 parasites μL^{-1} , $P < 0.05$).

RESULTS

MRR detection and quantification of cultured *P. falciparum* 3D7 iRBCs. We synchronized parasites from cultured blood to obtain highly enriched ring stage parasites free of any residual hemozoin, by using sorbitol treatment¹⁸ and magnetic separation¹⁹ (Methods and Supplementary Fig. 1). We prepared and measured 3 independent samples (A, B and C) with different purity of ring-stage iRBCs (Fig. 2a and Supplementary Fig. 2). The normalized $\Delta R_2 = R_{2(\text{iRBC})} - R_{2(\text{RBC})}$ indicate a strong correlation ($R^2 > 0.95$), for all three samples. $R_{2(\text{RBC})}$ represent the baseline correction of the uninfected RBCs.

Sample A recorded as little as 0.00025% parasitemia (12.5 iRBCs μL^{-1}), which corresponds to less than 10 iRBCs inside our probe (estimated ~ 300 nL active volume within the coil's detection region). Data for parasitemia level below 0.00025% ($p < 0.001$) were indistinguishable from uninfected RBCs. Sample B and Sample C recorded a limit of detection of 0.0002% ($p < 0.0003$) and 0.0007% ($p < 0.05$), respectively. The limit of detection of the current MRR setup is in the order of 0.0001% for *P. falciparum* infection. Importantly, samples A, B and C reflect the parasite stages observed in the peripheral blood of infected patients reasonably well²⁰ (Fig. 2a).

***P. falciparum* 3D7 detection and measurement in continuous culture.** We tracked the daily growth of *P. falciparum* 3D7 parasites in continuous culture and evaluated the R_2 -responses during the first few cycles. We inoculated healthy donor blood with highly synchronized ring stage parasites (94% R, 6% ET, 0% S) to obtain an initial parasitemia of 0.5 iRBCs μL^{-1} . We took 10 samplings per time point every 24 h (Fig. 2b). The R_2 -readings displayed a steady increase over time reflecting the increase in parasite levels in the culture. Furthermore, in every 24 h of interval, the late stages trophozoites have higher R_2 -readings as compared to its' rings counterpart for the same cycle (Fig. 2c).

Importantly, we observe that 48 hr (Day 2) after the initial inoculation in the first ring-cycle, the R_2 -readings showed a pronounced shift ($p < 0.0001$) from the control readings. Theoretically with an expected 5–10 fold increase per cycle¹⁸, by Day 2 the parasitemia levels would have grown up to about 3 iRBCs μL^{-1} (0.00006%). From 10 samplings, the mean readings for Day 2 was $(8.06 \pm 0.30) \text{ s}^{-1}$ as compared to the mean control readings of $(7.20 \pm 0.15) \text{ s}^{-1}$. In fact, the Receiver Operating Characteristic (ROC) on Day 1 is already indicating a good diagnostic

performance (Area Under the Curve (AUC) > 0.80) and an excellent diagnostic performance beyond Day 2 (Fig. 2d and Supplementary Fig. 3).

***In vivo* detection of *P. berghei* infection on BALB/c mice.** We used the murine malaria parasite *P. berghei*, which is a good model of human *P. falciparum* infection, for the *in vivo* validation of this approach²¹. We intraperitoneally infected BALB/c mice ($n = 25$) with $\sim 10^4$ *P. berghei* parasites (denoted as Day 0) (Methods and Supplementary Fig. 4a). The infection was allowed to establish itself for one day. From Day 2 to Day 13 post-infection, we analyzed tail blood from the mice each day both by MRR measurements as well as thin smear microscopy (Fig. 3a). *P. berghei* infections in laboratory rodents tend to be asynchronous. Therefore, the measured transverse relaxation rate, “ R_2 -index”, reflects the signal from a mixture of parasite stages, instead of the more synchronized ring stages of *P. falciparum* found in human host.

In general, R_2 -index of the mice and parasitemia levels increase with the number of post-infection days, with a slight fluctuation in R_2 -index being observed in the early days of infection (Fig. 3b). Reliable counting of parasitemia using Giemsa stained smears was, however, only possible from Day 6 onwards (Supplementary Table 2) and showed that the parasitemia levels gradually increased and reached approximately 25% by the end of the experiment on Day 13, consistent with the reports that BALB/c mice survive through the early phase of infection and eventually die of anemia and high parasitemia²². Remarkably, there is a strong linear relationship ($R^2 > 0.96$) between the measured R_2 -index and parasitemia, as determined by blood smear counts (Fig. 3c). ROC analysis was performed to investigate the accuracy of differentiating infected mice over uninfected mice throughout the experiment from Day 2 to Day 13 (Supplementary Fig. 5). We further established the limit of detection of *P. berghei* ANKA infection and found it to be as low as 0.0001% parasitemia (Supplementary Fig. 6—7).

Blinded Test: early stage of *P. berghei* infection. In order to evaluate the performance of MRR as a diagnostics test, we carried out a blinded study focusing on the early stage (Day 1 to Day 6) of *P. berghei* infection in BALB/c mice. We randomly infected BALB/c mice ($n = 58$) intraperitoneally with asynchronously mixed-stages of *P. berghei* parasites in three different sizes of inoculum ($\sim 10^7$, $\sim 10^4$, zero as control), blinded from the microscopists and MRR technician. Five experienced microscopists and a well-trained MRR technician were first asked to independently and rapidly screen for a parasites using both blood smear technique and MRR (Methods and Supplementary Fig. 4d). At the end of the experiment the results were cross-checked and classified as True Positive, True Negative, False Positive and False Negative (Table. 1).

Not unexpectedly, in the early days of the infection, blood smear evaluation performed poorly (average sensitivity of 74.9%, specificity of 82.0%, and accuracy of 75.5%), and great variability among microscopists (Fig. 4a). The accuracy of microscopy improved significantly after Day 3 of infection when parasitemia increased above 0.01% (Supplementary Table 5). In contrast, MRR measurements were demonstrating better diagnostic performance (97.9% sensitivity, 90% specificity, and 96.9% accuracy) (Table 1). Importantly, MRR has > 90% accuracy as early as Day 1, making it a viable tool that can complement the shortcoming of the current gold-standard.

Finally, the blood smear slides were once again cross-checked carefully without time-constraint for detailed quantitative analysis (Fig. 4b, Supplementary Figs. 8 and 9). Reconstructed R_2 -index against the post infection days mapped similar trends of parasitemia levels as evaluated by blood smear microscopy (Fig. 4c). The MRR measurements also provide new insights into pathophysiology of early post-infection days in the rodent mouse model (Figs. 3 and 4). For the first time we can show the existence of a critical event at around Day 4 post-infection (10^4 inoculum), where the median R_2 -index is seen to be slightly lower than the R_2 -index on Day 3. This result is consistently observed in multiple experiments and is also seen, albeit less pronounced and on Day 2 when a higher initial inoculum (10^7 inoculum) is used. Evidently, we observed that the parasite multiplication rate had slowed down by Day 4 and Day 3, for inoculum 10^4 and 10^7 , respectively (Supplementary Table 5d—5e).

DISCUSSION

The rapid, sensitive and reliable early detection of *Plasmodium* infected patients is a key component for the global effort to eliminate malaria. Here we have clearly demonstrated that the hemozoin crystallites, metabolic by-products of malaria parasite known to be present in all - stages and all human-infecting strains of malaria¹⁵, can be used as a natural biomarker for malaria diagnostics. A novel classification via magnetic susceptibility “ R_2 -index” measured with MRR technique correlates well with iRBCs load, which can be a valuable metric for clinical prognosis, monitoring drug resistance, hospitalization criteria, and assessing severity of infection.

The higher sensitivity of the MRR shown in this work can be attributed to a few factors. Firstly, the micron-sized radio-frequency detection coil, which produces strong rf-nutation field per unit current²³. Secondly, advances in microelectronics enable faster rf-switching and higher sampling rate of analog to digital converter, which has been critical for Ultra-short Echo Technique (UET)^{23,24} in MRR spectroscopy adopted here. This technique retrieves spin-echoes at very short echo time (10—100 μ s) over a long array of pulses, therefore increasing the signal to noise ratio of tissues/cells of very short relaxation rates or in the presence of strong

inhomogeneous field²⁴. We have shown that concentrating the RBCs via standard centrifugation and exploiting UET technique has given a signal sensitivity enhancement, compared with work reported by Karl *et al.* (Supplementary Fig. 10)²⁵.

The key features of the reported system includes, (i) highly sensitive label-free detection (< 10 parasites μL^{-1}), (ii) less prone to human-error, (iii) minimal blood volume (a few μL), (vi) minimal sample preparation, (v) field-deployable system, and (vi) ultra-low-cost per assay ($< \$0.10$). Given these advantages, we believe that MRR can be an attractive alternative to traditional malaria diagnostics such as Giemsa-blood smear microscopy and dipsticks. Currently, electronics and polarizing magnets are the main cost driver of the MRR system. Yet, we¹¹ and others^{8,26} have shown that an entire MRR system (along with inexpensive magnets) can be packaged onto a single chip pre-programmed with radio-frequency pulse sequences, which will make it cheaper (less than \$2000), smaller and easier to use in point-of-care settings^{8,26}.

Author's contributions:

W.K. Peng and J. Han conceived the original idea and designed the study, W.K. Peng built the whole experimental setup, designed the protocol and performed MRR measurements of *P. falciparum* and *P. berghei* study, L. Chen assists in rf-probe designing, C.S. Ng, W.K. Peng, A.A.S. Bhagat, and L. Chen involved in preparing *P. falciparum* parasites samples. T.F. Kong, C.S. Ng, W.K. Peng and Y. Huang worked on the *in vivo* mice studies (Day to Day measurements). C.S. Ng, W.K. Peng, L. Chen, and T.F. Kong, contribute equally to the blinded test mice studies. P.R. Preiser, N.T. Nguyen and J. Han supervised the mice works. W.K. Peng, P.R. Preiser, J. Han, T.F. Kong, and C.S. Ng wrote the paper and all the authors checked through the manuscript and analyzed the data.

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FIGURE LEGENDS

Figure 1

(a) A train of rf-pulses at the resonance frequency of 21.65 MHz with respect to the proton nuclei spins were applied at an inter-echo time interval, t_{echo} repeatedly for thousands of echoes.

(b) The transverse relaxation rate, R_2 measured is a function of the parasites load present in the sample. Due to the presence of highly paramagnetic hemozoin crystallites formed in the intra-erythrocytes cycle of the *Plasmodium spp.* infection, proton spin-spin interaction in iRBCs dephased much faster than the one in uninfected RBCs ($R_{2(\text{iRBCs})} > R_{2(\text{RBCs})}$).

(c) 48 h life cycle of *p. falciparum* during the intra-erythrocytic cycle, indicating the 4 distinctive stages; ring (R), early trophozoite (ET), late trophozoite (LT) and schizont (S). This pigment is initially deposited inside the vacuoles within the erythrocytes in the ring stage and subsequently become more prominent as the cell-cycle proceeds into the later stages.

(d) Schematic illustration of MRR system consists of portable permanent magnet that provides strong polarizing magnetic field, and home-built rf-detection probe. For clarity, the drawing for permanent magnet has been simplified (not according to scale). The biosensor is connected to a rf-spectrometer which acts as a transmitter and receiver of the rf-signal. A microcentrifuge is used to separate the plasma from RBCs-iRBCs pellet inside a microcapillary tube (Methods). The microcapillary tube is then slotted into the rf-detection probe. Rf-circuitry (shown in insert) of detection probe ($50\ \Omega$ impedance) was tuned to the resonance frequency for proton (21.65 MHz) using the variable capacitance at $C_t = 40\ \text{pF}$ and $C_m = 800\ \text{pF}$.

Figure 2

(a) Plots of enhancement of transverse relaxation rate, ΔR_2 as a function of parasitemia levels carried out on three independently cultured *P. falciparum* parasites blood sample A—C spiked with whole blood of three different healthy donors. The percentage numbers listed for each sample designate the fraction of ring (R), early trophozoite (ET), late trophozoite (LT), and schizont (S) , respectively. 5 samplings for MRR measurement were carried out. CPMG echo trains of 60 μ s interecho times were used. All data are shown in (means $\pm$ s.e.m). The baseline of uninfected control blood is indicated with grey-bar.

(b) Profiling of the R_2 -index against the days after culture synchronization every 48 hr (Methods). The box plots show the lower quartile, median, and upper quartile of their respective sample distributions. The control reading (grey color) denoted as C1 to C8 for its respective days. Inter-echo time of 200 μ s consisting of 2000 echoes, a total of 24 scans was used.

(c) Plot of transverse relaxation rate, R_2 as a function of theoretically 'expected' parasitemia levels based on the numbers of culture days. R_2 were means values of 10 samplings and the error bars were its' corresponding standard mean measurement. A zoom-in log—log plot is shown in the inset. For parasitemia of $> 0.01\%$, thick and thin-smear microscopy technique were used for verification purposes. 50 fields of more than 5000 RBCs were counted to estimate the parasitemia.

(d) ROC characteristics of the diagnostic performance of MRR system for day to day monitoring of culture growth. ROC curve is a plot between sensitivity (true positive fraction) against 100—Specificity (false positive fraction). The AUC values from Day 1 to Day 8 were (0.80, 1.00, 0.96, 1.00, 1.00, 1.00, 1.00, 1.00) as indicated by the side of each point. AUC value of 0.90—1.00 is considered excellent accuracy, 0.80—0.90 is good, 0.70—0.80 is fair, and below 0.60 is poor (Supplementary Fig. 3).

Figure 3

(a) A microscopy image of actual *P. berghei* parasitized blood extracted from the one of the infected mice on Day 10. A parasitemia level of about 10% of mixed stages was obtained.

(b) Profiling of the R_2 -index (left axis) and parasitemia level (right axis) against the post-infection days. The box plots show the lower quartile, median, and upper quartile of their respective sample distributions. In order to avoid anemia or other complications, 25 mice were grouped into five groups. Only one group (5 mice) was bled each day, and the rotation

continued until Day 13. The readings were taken from 5 different mice per time point. For each mouse, 6 repeated MRR measurements were carried out. The R_2 -index uncertainty due to mice variations (baseline correction) is indicated with grey-bar. For direct comparison, we indicate “ R_2 -index” which represents the absolute value of the transverse relaxation rate, instead of the normalized “ ΔR_2 -index”, as in the case of *P. falciparum* study.

(c) Plot of transverse relaxation rate, R_2 as a function of parasitemia levels in log-log plot format. Strong linear correlation ($R^2 > 0.96$) between the MRR measured, R_2 -index and the “parasite load” as evaluated by thin-smear microscopy technique ($>1\%$).

Figure 4

(a) Accuracy of the test with MRR and blood smear against the post-infection days. Accuracy-index range from 0 to 1, with 1 is very accurate and 0 is not accurate at all (Supplementary Table 5a—5b).

(b) Reconstructed R_2 -index obtained from all the 58 mice with IP-infected inoculum size of $\sim 10^7$ (green) and $\sim 10^4$ (blue) of *P. berghei* parasites against the post-infection days. The box plots show the lower quartile, median, and upper quartile of their respective sample distributions 4—8 samplings were carried out for the mice, and three MRR measurements were captured for each sampling (Supplementary Table 5 and Supplementary Fig. 8—9). The R_2 were measured by standard Carr-Purcell-Meiboom-Gill train pulses (200 μ s of interecho time) consisting of 4000 echoes. A total of 16 scans were typically acquired for signal averaging. A recycle delay of 2 s is used. The blood samples were prepared by using saponin-lysis method (Method).

(c) A log-log plot of transverse relaxation rate, R_2 as a function of parasitemia levels as evaluated by thin-smear microscopy technique (Supplementary Fig. 9). The error bars in x-axis were discrepancy between three microscopists (Supplementary Table 5a).

Table 1

A summary of the overall performance index for MRR as compared to blood smears microscopy, conducted blindly from Day 1 to Day 6 (details in Supplementary Fig. 8—9). As much as 50 fields (or > 15,000 RBCs) were checked for blood smear. The variables measured were the number of true positives (TP), number of true negatives (TN), number of false positives (FP), and number of false negatives (FN). Sensitivity was calculated as $TP / (TP + FN)$, specificity was calculated as $TN / (TN + FP)$ and Test accuracy is defined as $(TP + TN) / (TP + TN + FP + FN)$. With the assumption that all the desired mice which were IP-infected and were successful, and if the mean R_2 measured is much higher than the 75-percentile range of the baseline healthy mice and is statistically significant (p -value < 0.05) in student t -test, it is considered as True Positive. Otherwise, if the mean R_2 measured lies the below 75-percentile or is statistically no significant (p -value ≥ 0.05) in student t -test as compared to the baseline healthy mice, it is considered as True Negative. False Positive is the event where the mouse is detected with infection, but was not pre-infected. False Negative is the event where the mouse is detected as no infection, but was actually IP-infected (Supplementary Table 5).

Methods

Integrated sample preparation and detection (general). For parasite detection, highly-synchronized ring stage *P. falciparum* and *P. berghei* parasites (Fig. 1d, Supplementary Fig. 1a and 4) are mixed well at room temperature (RT) via repeated pipetting before being transferred into a microcapillary tube (Drummond Scientific Co.) via capillary action. Repeated pipetting ensures that any deoxygenated hemoglobin (deoxy-Hb), which is in the form of paramagnetic state is converted into oxygenated-hemoglobin (oxy-Hb) which is in the diamagnetic state, thereby creating a universal baseline for all other measurements^{16,17}. From our experience, the residual fraction of deoxy-Hb drawn directly from patients is easily converted to oxy-Hb upon exposure to the ambient air. It is also worth noting that the trace amount of methemoglobin (metHb) present in human blood physiologically is also in a paramagnetic state. The ferric state (Fe^{3+}) in metHb, however, cannot be reduced back to its original ferrous state (Fe^{2+}) through physical mixing such as pipetting. Since the current protocol is not designed to remove the trace amount of metHb in whole blood, any unusually high amount of metHb (methemoglobinemia) may introduce false-positive signal, or patient-to-patient variation.

Once the blood sample is transferred to the capillary tube, one end of the tube is sealed with an inert tube sealant (Critoseal, Krakeler Scientific Inc.) and the tube is centrifuged with a microcentrifuge (Sorvall Legend Micro 21) at 6000g for 3 minutes. The microcapillary tube is then transferred to the MRR detection coil (Fig. 1d). MRR detection was carried out on the concentrated (near 100% hematocrit) band of RBCs, which allows us to remove the possible confounding effect of variations in blood hematocrit number among individuals. This important strategy enhanced the MRR sensitivity drastically, in contrast to the work by Karl and coworkers²⁵, where centrifugation is not used. We discuss the effect of centrifugation on the of sensitivity detection (Supplementary Fig. 9).

For the case of *P. berghei* iRBCs, which have less density²⁷⁻³⁰ than non-infected RBCs, an additional step was added to lyse the cell and submerge the hemozoin to the bottom of the tubes, upon centrifugation. Two techniques can be used to lyse the cells; by using Giemsa incubation (Supplementary Fig. 4a) and saponin cell-lysis method (Supplementary Fig. 4d) can be used. The whole MRR assay, including both sample preparation (less than 5 minutes for the case of *P. falciparum* and less than 10 minutes for the case of *P. berghei*) and detection stage (less than a minute), can be done in approximately 10 minutes. In addition, the whole process is designed to minimize sample loss as well as avoid contamination and can easily be adapted to process multiple samples at the same time.

MRR measurement and detection (*P. falciparum*). We performed ^1H MRR measurements of bulk red blood cells at the resonance frequency of 21.65 MHz inside a portable permanent magnet (Metrolab Instruments, Switzerland), $B_0 = 0.5$ T with bench-top type console (Kea Magritek, New Zealand). We constructed a single resonance proton MRR probe with detection coil of 900 μm inner diameter to accommodate MRR sample microcapillary tube (o.d: 900 μm , i.d: 550 μm) (Drummond Scientific Co., Broomall, PA) for a detection region of approximately 300 nL. Otherwise, we used heparinized microcapillary tubes (o.d: 1500 μm , i.d: 950 μm) (22-260-950, Fisherbrand) for a 1 mm microcoil rf-probe (mentioned in the *Text*). We mounted the electronic parts and coil on a single printed circuit board (Fig. 1c). We measured the transverse relaxation rates, R_2 by standard Carr-Purcell-Meiboom-Gill train pulses (60 μs of interecho time) consisting of 5000 echoes. We acquired a total of 48 scans for signal averaging unless mentioned otherwise. We measured all samples at room-temperature. We acquired all data five times and reported as (means $\pm$ standard error measurement (s.e.m)). We maintained the transmitter power output at 12.5 mW for a single 90° -pulse of pulse length 14 μs , which correspond to nutation frequency of 17.9 kHz. We set a recycle delay of 1 s, which is sufficient to allow all the spins to return to thermal equilibrium, between each pulse. For experiments with magnetic beads suspended in DI water, we used longer recycle delay of 30 s. We used the same parameters throughout this work except otherwise mentioned in the *Texts*.

***In vitro* culture of *P. falciparum*.** We used *P. falciparum* 3D7 strain (MR4, USA) in this study. Parasites were cultured in RPMI medium 1640 (Invitrogen, USA) supplemented with 0.3 g of L-glutamine, 5g of AlbuMAX II (Invitrogen, USA), 2 g NaHCO_3 , and 0.05 g of hypoxanthine (Sigma-Aldrich, USA) dissolved in 1 mL of 1M NaOH, together with 1mL 10mg mL^{-1} of Gentamicin (Invitrogen, USA). We synchronized the parasites at ring stage using 2.5% D-sorbitol to maintain a synchronous culture. We incubated the cultures at 37 $^\circ\text{C}$ after gassing with a 5% CO_2 , 3% O_2 and 92% N_2 gas mixture and their hematocrit maintained at 2.5%. We harvested highly synchronized ring-stage iRBCs (more than 90%). We obtained whole blood from healthy donors and pelleted down the RBCs for parasite culture. We treated the RBC pellet with citrate phosphate dextrose adenine (CPDA) for 3 days before being washed three times with RPMI 1640 and stored for use.

Protocol to prepare highly synchronized ring-stage sample which mimics the actual patient's peripheral blood sample. We obtained *P. falciparum* infected RBCs of 10.0% parasitemia level from cultured parasites, as described above. We spun down these iRBCs in a microcentrifuge at 1,000 g for about 3 min, washed the pellet three times with isotonic PBS solution, and re-suspended in PBS solution (Supplementary Fig. 1a). The PBS solution was previously bubbled with ambient air for a few minutes so that all the hemoglobin will be converted into oxy-hemoglobin states. For each sample, we determined the relative ratios of parasite stages by standard Giemsa-stained blood microscopy. We prepared samples of various parasitemia levels

by spiking iRBCs into uninfected RBCs and subsequently diluted to various parasitemia levels ranging from 0.0002% to 6.5%.

Differential counting of Giemsa-stained blood smears of *P. falciparum* infected RBCs.

Differential counting of Giemsa-stained blood smears of the different parasite stages of the highly synchronized ring-stage culture (sample A) indicated 92.1% rings, 7.3% early trophozoites, 0.6% late trophozoites, and 0% schizonts³¹. We serially diluted the ring stage parasites with uninfected RBCs obtained from a healthy donor to obtain different final parasite concentrations (0%, 0.00025%, 0.0005%, 0.001%, 0.0025%, 0.005%, 0.01%, 0.05%, 0.1%, 0.5%, 1%, 5%). We measured the transverse relaxation rates of proton MRR, R_2 of the various infection levels, $R_{2(iRBC)}$, which reflect the instantaneous parasite load of the sample.

Magnetic separation. We further purified the parasite cultures which consist of highly purified ring stage¹⁹ with MACS system (25 LD columns, Miltenyi Biotec, Germany). We preloaded the LD columns with rinsing buffer (0.5% BSA in 1x PBS), which was held with Quadro MACS magnetic support. The blood was then passed through the column. The non-magnetic parts (uninfected RBCs) and iRBCs with low magnetic susceptibility especially the ring-stage will be able to pass through the column without being trapped. Most of the late stages (trophozoite and schizont) and suspending hemozoin from the previous generation will be trapped inside the column (Supplementary Fig. 1).

Protocol for day-to-day *P. falciparum* cell-culture growth with MRR measurement. We cultured *P. falciparum* 3D7 strain (MR4, USA) with the protocol as described above. We used D-sorbitol and magnetic separation techniques to produce highly synchronized ring-stage of culture. We spiked highly synchronized rings stage (94% ring, 6% early trophozoite, 0% schizont) into a healthy donor blood to form an initial parasitemia of 0.5 parasitized RBCs per μL ($0.5 \text{ pRBCs } \mu\text{L}^{-1}$), denoted as Day 0. We carried out magnetic separations every 48 h to maintain the synchrony and remove the gametocytes of the previous generation. We performed MRR measurements every 24 h (Fig. 2b). 10 samplings which consist of roughly 4 μL packed RBCs for the readings of each time point were taken. We used a heparinized tube (22-260-950, Fisherbrand) to sample out the blood from culture and performed standard microcentrifuge (3,000 g, 1 min) to separate the packed RBCs and the media. A microcoil probe (o.d: 1550 μm) which accommodate 4 μL volume of packed RBCs is used in this work.

MRR measurement and detection of *P. berghei* ANKA (Day to day growth monitoring). We extracted approximately 20 μL of whole blood from the mouse tail. We extracted uninfected blood samples from the healthy mice (as control), while extracting the infected blood samples from Day 2 upon *P. berghei* intraperitoneal-infection. We checked the hematocrit level of the sample by standard centrifugation technique. Blood extracted from mouse was mixed well in ambient air via repeated pipetting. We pipetted approximately 750 nL of RBCs via

microcapillary tubes and incubated it with 10 μ L of Giemsa solution for approximately 5 min. This is to allow all *P. berghei* parasitized RBCs which has density less than the uninfected RBCs^{27,28} to settle down in the same band upon centrifugation (Supplementary Figure 6). We then centrifuged the microcapillary tube at 6,000 g for 3 min to separate the plasma from the RBCs, and finally slotted the tube into the rf-probe for MRR measurements (Supplementary Figure 5). We carried out daily MRR measurements from Day 2 onwards. We performed¹H MRR measurements of bulk RBCs with the same parameters as that for *P. falciparum* experiments.

Mice and *P. berghei* ANKA parasite. Male BALB/c mice of 6–8 weeks old were obtained from Sembawang Laboratory Animal Center, National University of Singapore, and subsequently bred under specific pathogen free (SPF) condition at Nanyang Technological University Animal Holding Unit. We infected the donor mice with cryopreserved stocks of *P. berghei* ANKA strain by intraperitoneal injection. We carried out subsequent passages to maintain the parasites and we monitored the parasitemia levels by thin blood smears stained with Giemsa. For the experiment summarized in Fig. 3, we used a batch of 25 healthy mice ($n = 25$) to determine the control baseline of R_2 reading. We collected 20 μ L tail bloods with heparin (Sigma Aldrich, US) from one mouse by tail nick. We injected same batch of mice ($n = 25$) intraperitoneally with 10^4 *P. berghei* parasitized RBCs. We did the samplings as described from Day 2 to Day 13 post-infection, with five mice as a group each day. We quantified the parasitemia levels using thin blood films stained with Giemsa. We took at least 5 fields and microscopic analyses were performed by two different individuals. For Day 2 to Day 5, the parasitemia levels were too low to be determined accurately by thin-smear technique.

Early stage of *P. berghei* ANKA infection in mice (blinded test). Male BALB/c mice of 6–8 weeks old were obtained from InVivos Pte Ltd, Singapore, and subsequently bred under SPF condition at Nanyang Technological University Animal Holding Unit. We infected the donor mice with cryopreserved stocks of *P. berghei* ANKA strain by intraperitoneal injection. We carried out subsequent passages to maintain the parasites and parasitemia levels by thin blood smears stained with Giemsa. We used fifty eight healthy mice ($n = 58$) to determine the control baseline of R_2 reading (Main Text). We collected 30 μ L of tail blood with heparin (Sigma Aldrich, US) from one mouse by tail nick. We injected same batch of mice intraperitoneally with 10^4 ($n = 30$) or 10^7 ($n = 18$) *P. berghei* parasitized RBCs on Day 0. We did the samplings of ten mice per day by extracting 600–800 μ L of whole blood from each euthanized mouse via cardiac puncture, ranging from Day 1 to Day 6.

The extracted blood was tested blindly by MRR, and blood smear Giemsa-microscopy, simultaneously. We checked the hematocrit level of each mouse by standard centrifugation technique and resuspended into 26.6% hematocrit level (centrifugation 6,000 g for 1 min). We mixed 5 μ L of 1% Saponin (Sigma Aldrich) into 15 μ L of iRBCs to lyse the RBC membrane, while

leaving the parasites intact. We immediately extracted 20 μ L of blood with microcapillary tubes (22-260-950, Fisherbrand), and sealed with an inert tube sealant (Critoseal, Krakeler Scientific Inc.) to prevent further oxidation which may result in False Positive in MRR measurements. After incubation in Giemsa solution, *P. berghei* parasites submerged down into a single narrow band at the bottom of the microcapillary tubes upon centrifugation (3000 g for 1 min). Finally, we slotted the microcapillary tube into the rf-probe (o.d: 1550 μ m) for MRR measurements. We regulated the temperature of the MRR system at 26 °C. We carried out daily MRR measurements from Day 1 to Day 6. We examined Giemsa-stained thin blood smears under microscope to determine the parasitemia level. We checked up to 100 oil-immersion fields (or 15, 0000 RBCs) for negative confirmation and early days of post-infection.

Data analysis for Blind Test. We evaluated the performance of MRR against blood smear microscopy technique. The variables measured were the number of true positives (TP), number of true negatives (TN), number of false positives (FP), and number of false negatives (FN). Sensitivity was calculated as $TP/(TP + FN)$, specificity was calculated as $TN/(TN + FP)$, the positive predictive value (PPV) was calculated as $TP/(TP + FP)$, and the negative predictive value (NPV) was calculated as $TN/(FN + TN)$. With the assumption that all the Intraperitoneal-infection were successful, and if the mean R_2 measured is higher than the 75-percentile range of the baseline healthy mice and is statistically significant (p -value <0.05) in student t-test, it is considered as True Positive. Otherwise, if the mean R_2 measured lies below 75-percentile or is statistically not significant (p -value ≥ 0.05) in student t-test as compared to the baseline healthy mice, it is considered as True Negative. False Positive is the event where the mouse is detected with infection, but was not pre-infected. False Negative is the event where the mouse is detected as no infection, but was actually IP-infected.

Ethics statement. This study was carried out in strict accordance with the recommendations of the NACLAR (National Advisory Committee for Laboratory Animal Research) guidelines under the Animal & Birds (Care and Use of Animals for Scientific Purposes) Rules of Singapore. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Nanyang Technological University of Singapore (Approval number: ARFSBS/ NIE A002). All efforts were made to minimize the suffering.

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Fig. 1
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Fig. 2
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Fig. 4
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	MRR	Blood smear technique						
		1	2	3	4	5	Mean	Std. Dev
True Positive, TP	47	28	39	32	41	38	36	5.4
True Negative, TN	9	9	7	8	8	9	8	0.8
False Positive, FP	1	1	3	2	2	1	2	0.8
False Negative, FN	1	20	9	16	7	10	12	5.4
Sensitivity	97.90%	58.30%	84.70%	66.70%	85.40%	79.20%	74.90%	13.50%
Specificity	90.00%	90.00%	70.00%	80.00%	80.00%	90.00%	82.00%	10.00%
Test accuracy	96.60%	63.70%	79.30%	68.90%	84.40%	81.00%	75.50%	7.90%

Table 1
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