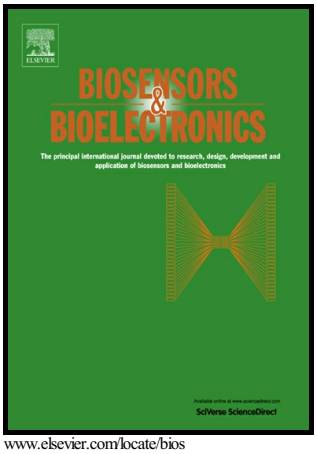

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A Portable Microfluidic Aptamer Tethered Enzyme Capture (APTEC) Biosensor for Malaria Diagnosis

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Abstract

There is a critical need for better biosensors for the detection and diagnosis of malaria. We previously developed a DNA aptamer that recognises the *Plasmodium falciparum* lactate dehydrogenase (PfLDH) enzyme with high sensitivity and specificity. The aptamer was integrated into an Aptamer Tethered Enzyme Capture (APTEC) assay as a laboratory-based diagnostic approach. However, a portable equipment-free point-of-care aptamer-mediated biosensor could have a significant impact on malaria diagnosis in endemic regions. Here, we present a new concept for a malaria biosensor whereby aptamers are coated onto magnetic microbeads for magnet-guided capture, wash and detection of the biomarker. A biosensor incorporating three separate microfluidic chambers was designed to enable such magnet-guided equipment-free colorimetric detection of PfLDH. A series of microfluidic biosensor prototypes were optimised to lower rates of inter-chamber diffusion, increase sensitivity, and provide a method for point-of-care sample testing. The biosensor showed high sensitivity and specificity when detecting PfLDH using both *in vitro* cultured parasite samples and using clinical samples from malaria patients. The high performance of the biosensor provides a proof-of-principle for a portable biosensor that could be adaptable for a variety of aptamer-mediated diagnostic scenarios.

Keywords

Aptamer, 3D print, Microfluidic, Magnetic Bead, Malaria, *Plasmodium falciparum*

1. Introduction

The global effort to eradicate malaria has seen mortality decline by 48% since 2000 (WHO 2015c). Notwithstanding, malaria remains endemic in 97 countries and caused an estimated 485 thousand deaths in 2015 (WHO 2015c). The WHO Global Technical Strategy for Malaria outlines universal access to diagnosis as essential to ensure the effective treatment of malaria (WHO 2015a). Point of care diagnostic tests decentralise the diagnosis of malaria for the many at risk populations which live in regions without access to good quality microscopic diagnosis (WHO 2015b) and have had a dramatic impact on improving clinical outcomes and disease surveillance. An estimated 314 million antibody-based malaria diagnostic tests were distributed in 2014 (WHO 2015b). Nevertheless, the existing antibody-based diagnostic tools have faced challenges due to cost (Cohen et al. 2015), instability in hot and humid conditions (Albertini et al. 2012; Jorgensen et al. 2006), poor characterisation of cross-reactive reagents (Marx 2013; Miller and Sikes 2015; WHO 2015b), and subjective test interpretation (Gillet et al. 2011; Seidahmed et al. 2008). These limitations contribute to the overuse of malaria drugs and inaccuracies in disease surveillance (WHO 2015c). Therefore, development of improved low-cost point-of-care diagnostics is required to address the challenges faced with current diagnostic tests used in malaria endemic regions.

Aptamers consist of single stranded oligonucleotide polymers. Aptamers with high specificity and high affinity are selected using the *in vitro*-evolution technique called Systematic Evolution of Ligands by Exponential Enrichment (SELEX) (Ellington and Szostak 1990; Tuerk and Gold 1990). These aptamers can then be used as bio-recognition agents for biomarkers ranging from small molecules (Liu et al. 2009; Stojanovic et al. 2000) to single cells (Morris et al. 1998; Shangguan et al. 2006). We previously demonstrated that the 2008s aptamer binds to a well-established diagnostic biomarker *Plasmodium falciparum* lactate dehydrogenase (PfLDH). The high specificity of aptamer 2008s for PfLDH over human LDH was confirmed by the PfLDH:2008s crystal structure, which revealed 2008s integrated within a PfLDH loop absent in human LDH (Cheung et al. 2013). Since the initial discovery of the 2008s aptamer, it has been successfully integrated into DNA origami (Godonoga et al. 2016), tweezers in the split form (Shiu et al. 2016), silver nanoclusters (Wang et al. 2017) and has also been enhanced using aptamer affinity maturation (Kinghorn et al. 2016). Aptamers offer significant benefits when compared to antibodies used in immunological diagnostics. These benefits include low-cost, high thermal stability, ease of chemical modification, consistent batch to batch generation and greater specificity (Jayasena 1999). The intrinsic activity of PfLDH makes it an ideal biomarker for integration into diagnostic tests, such as the Malstat assay (Makler and Hindrich 1993; Markwalter et al. 2016), which immunologically captures PfLDH and uses its intrinsic enzymatic activity to trigger a colour change in a spectroscopically detectable tetrazolium dye. We used the 2008s aptamer to replace the antibody-capture component of Malstat assay, resulting in the highly stable lab-based Aptamer Tethered Enzyme Capture (APTEC) assay (Dirkzwager et al. 2015).

The availability of low-cost high precision 3D printers (Berman 2012) has spurred microfluidic fabrication (Bhattacharjee et al. 2016) and the development of microfluidic biosensors, for the detection of small molecule biomarkers (Chan et al. 2016; Gowers et al. 2015; Roda et al. 2014) and pathogenic bacteria (Lee et al. 2015; Lee et al. 2014). The potential benefits of 3D printed fabrication include customised device design (Tsuda et al. 2015), simplification of multistep assay protocols (Ho et al. 2015), integration with tele-medical technologies (Chan et al. 2016; Roda et al. 2014), a wider choice of fabrication materials (Tsuda et al. 2015), and lower barriers to translate technology from the lab to commercial applications (Waheed et al. 2016). Most notably, 3D printing allows for the decentralisation of prototype manufacturing, as designs can be distributed online and printed anywhere in the world. This decentralisation is mirrored by aptamer technologies, as aptamer sequence data can be electronically distributed and aptamers synthesised anywhere in the world.

The 96 well plate APTEC assay is lab-based and must be quantified spectrophotometrically. Since its publication the APTEC assay has been developed into portable diagnostic tests proving the principle that the APTEC could be integrated into a 3D printed devices for use at the point-of-care (Dirkzwager et al.

2016). The APTEC device developed had several limitations including: the use of large sample and solution volumes; the device was not enclosed and therefore exposed the end-user to blood samples; and lacked sensitivity when tested with parasite blood cultures. Here, we present a next generation microfluidic diagnostic platform optimised for greater functionality with patient blood samples and focused to the requirements of malaria endemic regions. Furthermore, we applied recent developments in telemedicine (Chan et al. 2016; Martinez et al. 2008a; Vashist et al. 2015), and developed a stringent, mobile detection system for objective quantification of the PfLDH positive purple signal. The resulting biosensor detected *P. falciparum* with high specificity and sensitivity from *in vitro* parasite cultures and clinical samples from malaria-infected patients.

2. Materials and Methods

2.1 Materials and instruments

Biotinylated 2008s aptamers were synthesised by Integrated DNA Technologies (U.S.A.): 5'-biotin- CTG GGC GGT AGA ACC ATA GTG ACC CAG CCG TCT AC -3'. Dynabeads® MyOne™ Streptavidin T1, ThermoFischer Scientific (U.S.A.). Phosphate Buffer Saline (PBS), Tween- 20, Triton X100, 3-acetylpyridine adenine (APAD), Nitrotetrazolium blue chloride (NBT), 1-Methoxy-5- methylphenazinium methyl sulfate (MePMS), Sodium-L-Lactate, Sigma Aldrich (U.S.A). Recombinant PfLDH was expressed in *E.coli* strain BL21(DE3) PLysS and purified with affinity chromatography HP 1mL column (GE healthcare) as described previously (Cheung et al. 2013). Human blood was obtained from the Australian Red Cross Blood Service and. blood samples from returned travellers with malaria were obtained from the Victorian Infectious Diseases Laboratory, Melbourne (Australia). Telemedical setup included iPhone 6 and iPad (1st Generation), Apple (U.S.A). Absorbance spectra were measured using The POLARstar® Omega Plate Reader Spectrophotometer from BMGlabtech (Germany). Figures were developed using 'Adobe Photoshop' 'Adobe Illustrator', 'Pymol' and 'Origin Pro'.

2.2 Aptamer coated magnetic beads

2 mg of Dynabeads® MyOneTM Streptavidin T1 were incubated in 200 µL of 2.5 µM biotin modified aptamer for 2 hours at room temperature, washed three times in wash buffer (PBS, 0.1% Tween-20), and stored in a concentration of 10 mg mL^{-1} in PBS/Tween at 4 °C. Magnets were actuated with nickel Plated Neodymium 12.7 mm height by 12.7 mm base diameter cone magnet, SuperMagnetman (U.S.A.).

2.3 Preparation of portable APTEC biosensor

The layout of the microfluidics device is shown (Fig S1). Two layers were printed by Freeform Pico in PlasCLEAR resin, Asiga (USA) and washed in isopropanol and ethanol to remove excess uncured resin. 5 µL of the parasitised blood sample was added to 5 µL of lysis buffer (2X PBS, 0.5 % Triton-X100) and then pipetted into incubation chamber with 80 µg magnetic beads were added into the incubation chamber. 25 µL PBS/Tween is pipetted into wash chamber and 20 µL of development solution pipetted into the development chamber. Development solution is freshly prepared as a 500uL stock: 25 µL NTB (10 mg mL^{-1}), 6.6 µL APAD (50 mg mL^{-1}) and 1 µL MePMS (5mg mL^{-1}) made to 500 µL with L-Lactate Buffer (0.2M sodium L-Lactate, 100 mM Tris-HCl, 0.2% Triton x-100, pH 9.1). Gelseal, from GE healthcare (U.S.A) was used to adhere 3D printed parts. after introduction of the reagents. All CAD files were designed on Autodesk Inventor 2016 and exported as 'stl' files.

2.4 Image analysis

The biosensors were placed on top of a first-generation iPad, which displayed a homogenous white light (Fig 2). The iPad and biosensors were covered by an opaque box and imaged by an iPhone 6. Video recordings were taken and images were captured specific time intervals (0 min, 3 min, 7.5 min, 15 min, 30 min, 60 min 90 min, 120 min, 150 min and 180 min). Time interval images were uploaded and analysed with 'ImageJ' software. A negative control normalised image scale on 'ImageJ' software and image exposure on 'Preview App OSX'. The add-on 'threshold colour' was used to select pixels of a specific colour channel. The 8-bit method was also performed as previously described (Dirkzwager et al. 2016). The protocol and equations for image quantification methods are outlined (Fig.S6. and Eq. S1).

2.5 Parasite culture

P. falciparum 3D7 parasites were cultured in RPMI media with 0.5 % AlbuMAX®, 0.2% sodium bicarbonate at 4% haematocrit, as previously described (Persson et al. 2006). Cultures were synchronised to ring stage by sorbitol treatment. Parasite stage and parasitaemia were confirmed with light microscopy using Giemsa stained blood slides and flow cytometry (BD FACS CANTO II). Cultured parasites were diluted with uninfected human blood samples to achieve a range of parasite densities and then diluted with heat-inactivated human serum to achieve a physiological haematocrit of 40%.

2.6 Storage and preparation of clinical samples

Whole blood samples were obtained travellers returning from overseas with malaria. These sample were speciated and had the parasite density calculated by expert microscopy at the Victorian Infectious Disease Reference Laboratory. These samples were de-identified and stored at -80 °C. All testing was performed blinded to the species and parasite density result. Ethics approval was obtained for the use of these specimens from the Alfred Hospital Ethics Committee (project number 84-13) and Melbourne Health Human Research Ethics Committee (project number 2004.038).

3. Results and Discussion

3.1 APTEC assay within a 3D printed microfluidic biosensor

The APTEC biosensor is a 3D printed microfluidic diagnostic test which uses small sample and reagent volumes, 5 µL and 20 µL respectively. The microfluidic device is composed of two separate layers, which were printed using stereolithography (STL) in a transparent biocompatible photopolymer (Waheed et al. 2016). The top layer contains solution entry-ports and the bottom layer contains a network of chambers and channels. All chambers and channels are sub-millimetre in height and therefore referred to as microfluidic (Fig. S1). A gel was spread between the 3D printed layers to create leak-proof channels, prevent solution evaporation and limit end-user exposure to hazardous blood samples. The assay was performed within a network of three distinct chambers connected by microfluidic stop valves, which lower the rate of diffusion between chambers (Fig. 1). Micro-magnetic beads were internalised within the device and as described in previous literature, actuated with an external magnet as a solid mobile phase (Gottheil et al. 2014). The operation of the biosensor consists of three stages. (i) Incubation: the mobile phase is incubated in a lysed sample of human blood. If positive for *Plasmodium falciparum,* aptamer-coated micro-magnetic beads bind specifically to the parasite protein PfLDH (Fig. 1a). (ii) Wash; the mobile phase is moved from the incubation chamber to the wash chamber, which functions to separate non-specific blood components from the mobile phase (Fig. 1b). (iii) Development; the micro-magnetic bead-aptamer-PfLDH mobile phase terminates in the development chamber, which contains the development reagent. As outlined in the *P. falciparum* specific reaction scheme (Makler and Hindrich 1993), an insoluble purple coloured formazan dye (NBT-DF) is developed in the presence of PfLDH, healthy human blood samples do not develop a colorimetric signal in the described assay (Fig. 1c). Malaria biosensors based on antibody-based lateral flow tests typically develop within 5-20 minutes (Murphy et al. 2013). The present biosensor developed a detectable signal within 20 minutes of development, but to ensure maximum sensitivity we allowed the development reaction to run for 180 minutes per biosensor cassette. Retail prices for commercial malaria tests range from 0.2 to 1USD (Jimenez et al. 2017). Based on a previous price analysis of our 3D printed prototype biosensors we estimate the cost of the present prototype biosensor to be ~3USD per cassette (Dirkzwager et al. 2016).

3.2 Microfluidic stop valves substantially decreased rates of diffusion

Stop valves were designed and prototyped for integration into the microfluidic biosensor to control diffusion rate. Stop valves are formed by decreasing width of a channel then sharply widening it, and have been used previously in PDMS microfluidics, for solution control (Zimmermann et al. 2008) and biological assays (Gottheil et al. 2014). To determine the most effective valve dimension we rapidly prototyped several diffusion test platforms. The platforms consisted of two chambers connected by a stop valve. The top chamber contained a low viscosity blue dye and the bottom chamber contained distilled

MilliQ water (Fig. S2a). The rate by which the blue dye diffused into the distilled MilliQ water was quantified using camera phone image analysis. A variety of stop valve conformations were printed and compared. A channel width of 200 µm (Fig. S2b), the limit of the 3D printer's lateral resolution, substantially decreased diffusion rate and allowed the chambers to be filled more precisely (Fig. S2b). A video of different diffusion rates of these prototypes is also available (Fig S3).

3.3 Optimisation of aptamer functionalised bead surface area for PfLDH capture

Single-stranded 2008s DNA aptamers were conjugated to micro-magnetic beads by streptavidin-biotin linkages forming a thermostable biomarker capture reagent (Fig.S4). The concentration of aptamer conjugated micro-magnetic beads within each test was optimised for maximum sensitivity in the APTEC assay. A greater aptamer-bead concentration was predicted to tether a greater number of PfLDH enzymes, which when subsequently transferred to the development reagent would generate a stronger colorimetric signal (Fig. S5a). The optimal amount of micro-magnetic beads required for PfLDH capture in the assay was determined. Several amounts of beads, from 0 to 80 µg, were incubated with recombinant PfLDH (100 ng/mL), then washed and transferred into the development reagent. The colorimetric signal generated was quantified by UV-Vis spectroscopy at 570nm. A positive correlation between bead mass and assay response was observed (Fig. S5b). A micro-magnetic bead concentration of 80µg was chosen for use to assess responses from *P. falciparum* parasite cultures and patient blood samples using the microfluidic APTEC assay.

3.4 Sensitive detection of cultured *P. falciparum* parasites using a mobile detection system

A series of six microfluidic assays (i.e. 1 negative control and 5 samples) were setup simultaneously on the iPad platform to determine the colorimetric signal (Fig. 2a). The setup included a uniform white LED screen, a light-shielded box, and camera phone (Fig. 2b). Mobile-cellular phones are integral and widely used tools in developing countries (Division 2016; Ondiege 2010; West and Chew 2014) and the efficacy of camera phone technology has been demonstrated extensively for healthcare applications (Abdur Rehman et al. 2016; Boulos et al. 2011; Chan et al. 2016; Dirkzwager et al. 2016; Fraser et al. 2004; Martinez et al. 2008a; Sarasohn-Kahn 2010; Vashist et al. 2015). The use of a mobile-cellular camera phone in this detection system was capable of capturing images coupled with metadata containing time, date, and GPS coordinates (Fig. S6); epidemiologically relevant data which could be used to monitor and forecast malaria prevalence. We established the efficacy of the microfluidic APTEC biosensor within the mobile detection system by testing human blood infected with synchronous ring stage *P. falciparum* 3D7 across a range of parasite densities. Based on previously described protocols we measured development signal pixel intensities (PIs) from 8-bit (greyscale) images (Chan et al. 2016; Dirkzwager et al. 2016; Martinez et al. 2008b; Vashist et al. 2015). This method did not effectively detect signal change for this device (Fig. 2c). This was in part due to the non-homogenous solution profile, found within the development chamber, a result of a high bead to reagent ratio. As an alternative approach, we adapted from a method originally used to measure pigmentation areas on leaves (O'Neal et al. 2002). This approach calculated the pixel area (PA) that contained the purple formazan dye (Fig. S7). We observed that the PA measurements were substantially more stringent than that of the 8-bit pixel intensity method (Fig. 2c). The lowest parasite concentration detected on the microfluidic biosensor using PA measurements was 250 parasites/µL (Fig. 2c). This is within clinical detection range (Jang et al. 2013). (Jimenez et al. 2017), and is substantially more sensitive than previous APTEC measurements. The lowest concentration of synchronous ring stage parasites detected on the 96 well based APTEC assay was at a concentration of 3,500 parasites/µL (Dirkzwager et al. 2015). The WHO Malaria RDT Testing Program recommends that tests achieve a panel detection score (PDS) of 75% for *P. falciparum* samples containing 200 parasites/µL (WHO 2015b). At this parasite concentration thirteen independent Rapid Diagnostic Tests (RDTs) have been shown independently to have PDSs ranging from 77%-96% (Jimenez et al. 2017). Based on the sensitivity of the present biosensor we believe that with further optimization of the biomarker capture reagent there is potential for it to outperform the 'best-in-class' RDTs (Jimenez et al. 2017).

3.5 Sensitive and specific detection of *P. falciparum* from patient samples

To fully establish the efficacy of the optimized microfluidic biosensor we tested clinical samples from travelers returning to Melbourne, Australia diagnosed with malaria. We compared light-microscopy estimates of parasite density with the findings of the microfluidic biosensor. These samples included samples from patients with *P. falciparum* (n=10), *P. vivax* (n=10) and negative controls (n=10). The biosensor assay performance was in agreement with sensitivities observed in parasite culture experiments with sensitivity of 90% across all patient samples. Notably it detected a sample parasitaemia lower than 0.01%, but failed to detect a sample at >0.001% parasitaemia (Table. S1). The test was also able to distinguish *P. falciparum* from *P. vivax* samples and non-infected blood samples, with a specificity of 90% across all patient samples. Two *P. vivax* malaria false positives were recorded and a *P. falciparum* malaria false negative (Fig. 3).

4. Conclusion

The optimisation of microfluidic chambers, stop valves, micro-magnetic bead concentration and the use of a mobile system for objective and stringent system colorimetric signal detection allowed for the detection of *P. falciparum* with high sensitivity and specificity in parasite culture and patient samples. The microfluidics chambers used fewer development reagents and a smaller blood sample whilst improving the sensitivity of the thermostable APTEC assay. We observed two false positive results from patient samples, to address this we will aim to test the aptamer against a larger number of clinical samples to determine its true specificity. We aim to develop a multiplexed biosensor using a Pv or Pan specific LDH aptamer. We will also aim to increase the sensitivity of this biosensor to a sub-microscopic level by improving the capture capacity of the aptamer coated magnetic microbeads.

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Conflicts of Interest

Authors JAT and YWC declare inventorship of patent US 9000137 B2 relating to the underlying aptamer used in this work.

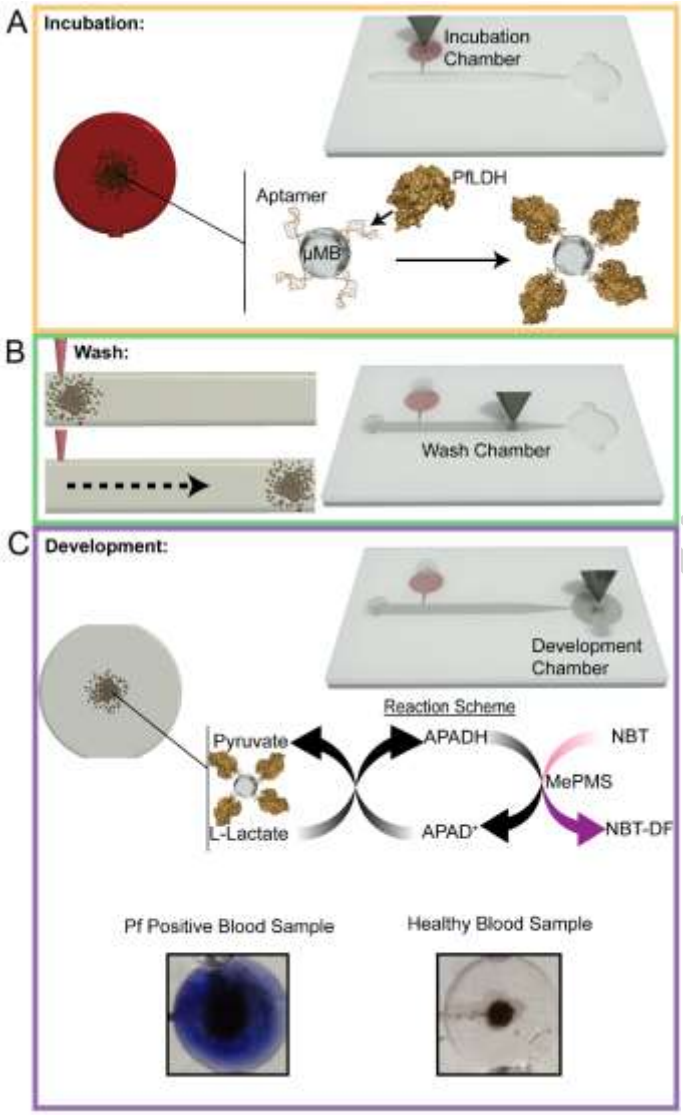

Fig.1. Operation and detection stages of the microfluidic APTEC biosensor. (A) Incubation Stage: Micro-magnetic beads (µMBs) are incubated in human blood. Unbound *Plasmodium falciparum* lactate dehydrogenase (PfLDH) (PDB 1T2C) binding to the 2008s aptamer forming a bead-aptamer- PfLDH complex (aptamer-PfLDH:PDB 3ZH2). (B) Wash Stage: The beads are magnetically actuated through the wash solution and are separated from non-specific blood components. (C) Development Stage: The beads are magnetically moved to the development solution which turns purple in the presence of PfLDH. The reaction scheme represents the reagents and redox reaction that result in generation of insoluble purple diformazan dye. Images show a result from a blood sample positive for *Plasmodium falciparum* malaria and a negative result from a healthy blood sample.

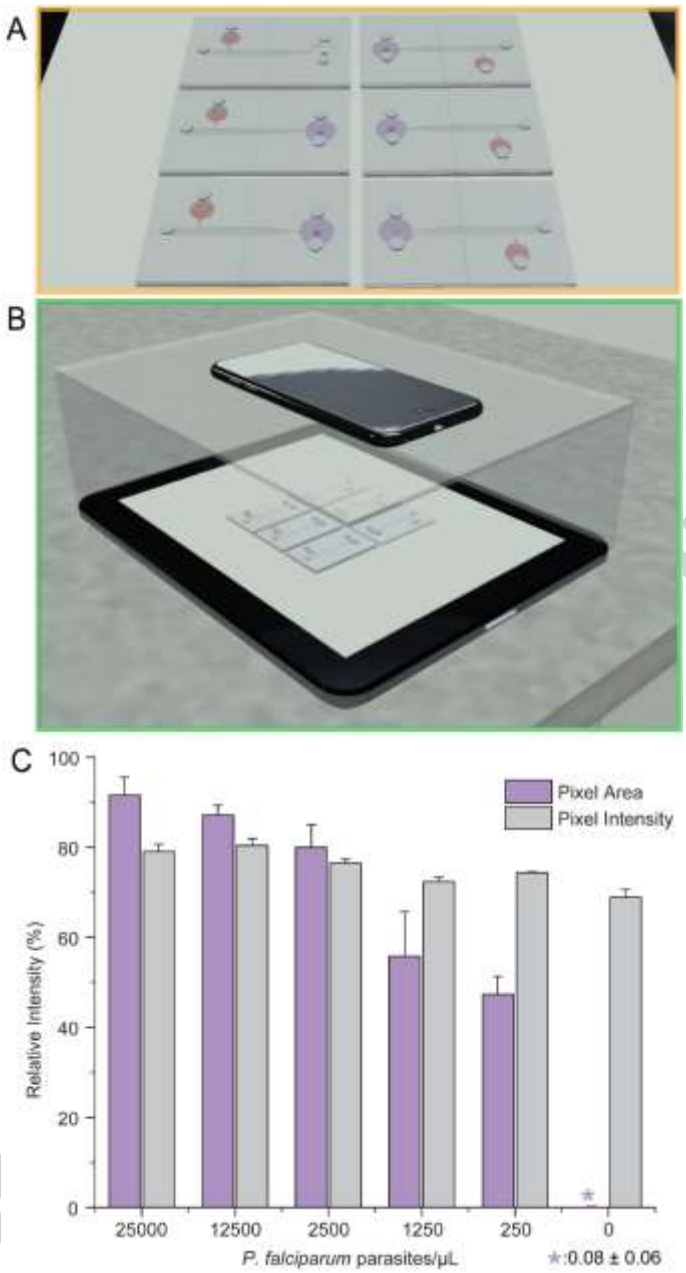

Fig.2. The mobile sample analysis method. (A) The setup of six biosensors illuminated by an iPad, one negative control assay (top left) and five sample assays. (B) Mobile tele-medical setup. An iPhone 6, placed above a light shielded box illuminated by an iPad, viewed development chambers of six microfluidic assays. (C) Comparison of colorimetric quantification protocols. The microfluidic assays were tested against range of parasite concentrations. Samples consisted of 5 µL of human blood. Infected samples contained synchronous ring-stage *Plasmodium falciparum* 3D7. The negative control, 0 parasite/µL samples were composed of healthy human blood.

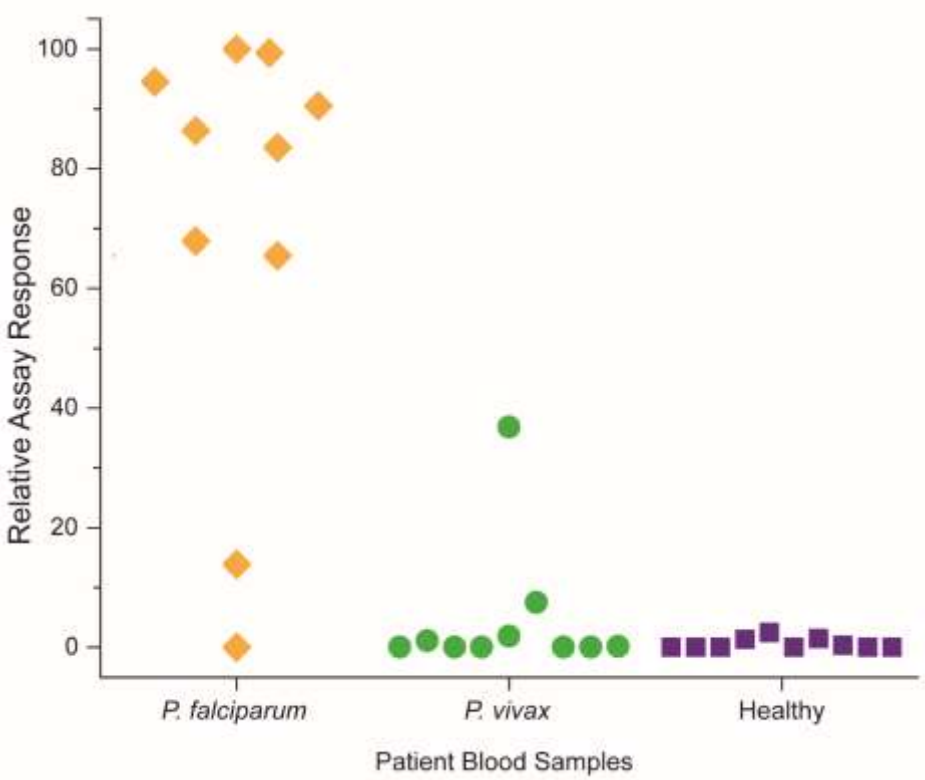

Fig.3. Tests on clinical blood samples provided by travellers returning from malaria endemic regions. The fully optimised diagnostic biosensor tested patient blood samples (5 µL of blood per test). The samples provided were either healthy or were infected with either *Plasmodium falciparum* or *Plasmodium vivax*.

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Highlights

- An aptamer-mediated magnet-guided microfluidic biosensor is developed.
- The biosensor can be visualized by eye without other equipment.
- The biosensor is integrated into a mobile tele-medical setup.
- The biosensor could detect *Plasmodium falciparum* infection in patient blood samples.