A RAPID POINT-OF-CARE TEST FOR MALARIA DIAGNOSTICS IN ELIMINATION SETTINGS

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Abstract

One of the grand challenges in science and engineering today is the development of innovative technologies to address global health concerns in resource-limited settings. This thesis aims to develop self-driven microfluidics-based diagnostic tools suitable for detecting malaria at the point-of-care (POC). In particular, the objective is to engineer devices that offer high sensitivity, short turnover time, and are amendable to mass manufacturing.

In this thesis, we examine the current diagnostic needs for malaria elimination, and identify gaps and opportunities in existing detection tools for malaria. Next, we provide an overview of new malaria diagnostic technologies that could be suitable for low resource settings. We also examine how innovative technologies such as microfluidics can assist in the global defeat of this ancient scourge. Specific examples are also discussed to illustrate the potential of advancement in technology in compliance to simplicity of use, appropriateness and affordability.

We develop microfluidics-based proof-of-concept diagnostic devices that are based on the understanding of an ideal malaria rapid test for the elimination context. Two key challenges are addressed, 1) the improvement of sensitivity, and 2) the integration of an immunodiagnostic assay into a self-driven microfluidics platform. To solve the first challenge, we use immuno-gold silver-staining (IGSS) as a mean to strongly amplify signal intensity. The growth of silver film over time for a model immunoassay is characterized and the optimum development time is in agreement with the literature. Additionally, we also confirm the stability of the assay using IGSS at 24°C and 37 °C.

To solve the 2nd challenge, we integrate a number of microfluidic elements on the chip. A highlight of this proof-of-concept is the elegant implementation of fluorescent microbeads that serves a two-fold purpose. First, the beads provide sufficient surface to immobilize capture antibodies (cAbs). Second, as the target analyte is captured, silver is produced and this silver film masks the fluorescence emitted from the core of the beads. The detection method for the device therefore relies on fluorescence attenuation and the analyte
concentration being inversely proportional. The integrated, capillary-driven microfluidic chip can accommodate 700 nanoliter of liquids and take 20 minutes to detect 17.1 ng mL\(^{-1}\) rabbit IgG, compared to milliliters of liquids and hours needed to detect 24.6 ng mL\(^{-1}\) rabbit IgG using a standard enzyme-linked immunosorbent assay (ELISA) on microtiter plates.

The proof-of-concept is further extended to detect the presence of Plasmodium \textit{falciparum} histidine-rich protein-2 (P\textsubscript{f}HR2) in human serum. Amongst various types of malaria Plasmodium infections, Plasmodium \textit{falciparum} (P\textsubscript{f}) infection is the most fatal one. Symptoms associated with P\textsubscript{f} infections are often misinterpreted with other febrile fevers, which results in available treatments not being administered in time. Therefore, the ability to detect the presence of P\textsubscript{f}HRP2 at the onset of infection is key. Current malaria rapid tests cannot detect low P\textsubscript{f}HRP2 concentrations (6 ngmL\(^{-1}\)), with the exception of nucleic amplification technologies. We base our work on the same chip design and fabrication procedure as before. Unlike the previous assay developed for rabbit IgG, we further improve the sensitivity of the P\textsubscript{f}HRP2 detection immunoassay by using secondary antibodies to amplify signal intensity before applying the IGSS technique. Since the core of the microbeads is fluorescent, photobleaching and stability issues often found in fluorescent assays are mitigated. The self-driven microfluidic chip is capable of achieving a limit of detection of less than 6 ng mL\(^{-1}\) P\textsubscript{f}HRP2 in human serum within 20 minutes. This limit of detection surpasses the requirement needed for the ideal malaria diagnostic tests for the elimination context set out by the Foundation for Innovative New Diagnostics (FIND).

Looking towards the future, such chips, especially if manufactured in low cost plastic and used in combination with smartphone-based fluorescence readers, have the potential to drive the widespread adoption of fluorescent bead-based immunoassays using capillary-driven microfluidics for POC diagnostics applications.

\textit{Keywords:} malaria, elimination, \textit{Plasmodium falciparum} histidine-rich protein-2, fluorescent, microbeads, silver staining, immunoassay, microfluidics, rapid diagnostic tests,
Un des plus grands défis en science et ingénierie de nos jours est le développement de technologie innovatrices pour adresser les problèmes de la santé publique dans des milieux de faibles ressources. Cette thèse a comme but de développer un outil diagnostique microfluidique autotractée capable de détecter la malaria sur le point de service. En particulier, l’objet est de former un dispositif qui offre une sensibilité très élevée, un temps de réaction courte et qui s’apprête pour une production en masse.

Pour commencer, nous examinons les besoins actuels pour l’élimination de la malaria et identifions des défauts et des opportunités dans des outils existants de détection de la malaria. Puis, nous donnons une vue d’ensemble du progrès technologique récent dans la détection qui puisse servir dans les milieux de faibles ressources. Nous examinons aussi comment des technologies innovatrices comme les puces microfluidiques peut assister l’éradication globale de cet ancien fléau. Des exemples spécifiques sont aussi analysés pour illustrer le potentiel de l’avancement de la technologie en adhérant aux principes de simplicités d’utilisation, de pertinence et de l’abordabilité.

Dans cette thèse, une démonstration de faisabilité d’un outil diagnostique basé sur des principes microfluidiques est développé basée sur la compréhension des capacités idéales d’un test rapide dans les conditions d’élimination. Les deux défis principaux sont surmontés, ce qui inclut une augmentation de la sensibilité et le transfert d’un test immuno-diagnostiques sur une plateforme microfluidique autotractée. Pour résoudre le premier défi, nous utilisons la technique du marquage immunogold argenté (IGSS) comme moyen d’amplifier fortement le signal. La croissance d’un film argenté en fonction du temps est caractérisée par un essai immuno-diagnostique modèle et le temps optimale de développement ainsi trouvé est en accord avec la littérature. En plus, nous confirmons la stabilité de l’essai IGSS à 24°C et 37°C.

Pour faciliter l’essai autotractée, nous intégrons un nombre d’éléments microfluidiques sur un puce. Le clou de la démonstration de faisabilité est l’implémentation élégante des micro perles fluorescentes qui mènent deux
buts distincts. D’abord, les perles fournissent une surface suffisante pour immobiliser des anticorps de capture (cAbs). Puis, quand l’analyte cible est capturé, un film argenté est formé qui atténue la fluorescence émit par du noyau des perles. Ainsi, la méthode de détection se base sur la l’atténuation fluorescente et la concentration d’analyte étant inversement proportionnelles. Ce puce microfluidique autotracté intégral peut accueillir 700 nano litres de liquide et prend 20 minutes pour détecter 17.1 ng mL\(^{-1}\) lapin IgG, en comparaison d’un millilitre de liquide et une heure requis pour détecter 24.6 ng mL\(^{-1}\) lapin IgG en utilisant un essai standardisé d’immuno-absorption enzymatique sur des plaques de micro titrage.

La démonstration de faisabilité est étendue pour des infections de la malaria en détectant la présence de la Plasmodium falciparum protéine-2 riche en histidine (PfHRP2) dans un sérum humain. Parmi plusieurs types d’infection Plasmodium de la malaria, celles de Plasmodium falciparum (Pf) est une des plus mortelles. Les symptômes associés avec une infection Pf est souvent confondu avec ceux d’une autre fièvre fébrile, ce qui a comme résultat que les traitements disponibles ne sont pas administrés à temps. Ainsi, la capacité de détecter la présence de PfHRP2 au début d’une infection est cruciale. Les tests rapides de malaria existants ne peuvent pas détecter de faibles concentrations de PfHRP2 (moins de 6 ng mL\(^{-1}\)), à l’exception de l’amplification d’acides nucléiques. Nous utilisons la même forme de puce et méthodes de productions comme auparavant pour l’essai avec lapin IgG. Contrairement au premier cas, nous amplifions l’intensité du signal d’avantage en appliquant des anticorps secondaires avant d’utiliser la technique IGSS. Puisque le noyau des perles micro sont fluorescentes, le photo blanchiment et des problèmes de stabilités qui se retrouvent souvent dans des tests fluorescents sont facilement évités. Ce puce microfluidique à deux couches, autotracté est capable de détecter toutes les densités de PfHRP2 supérieur à 6 ng mL\(^{-1}\) dans un sérum humain dans 20 minutes. Cette limite de détection répond ou même surpasse les exigences requises pour le test diagnostique de la malaria idéal comme imposées par la fondation Innovative New Diagnostics (FIND) pour la phase d’élimination de la malaria.

En conclusion, de tels puces microfluidiques tractés par des forces capillaires, basés sur des tests IGSS avec des perles micro peuvent servir pour détecter plusieurs marqueurs biologiques en parallèle et en plus peuvent être combinés avec un système de lecture basé sur smartphone pour obtenir une plateforme diagnostique idéale au point de service (POC) et un jour ou l’autre aboutir à la diagnostique clinique décentralisée de plein de maladies infectieuses.
Mots clés : malaria, élimination, Plasmodium falciparum protéine-2 riche en histidine (PfHRP2), fluorescence, micro perles, coloration à l'argent, immuno-essai, microfluide, test diagnostique rapide
Acknowledgements

This thesis would not have been possible without the mentorship and continuous support of my supervisor Emmanuel Delamarche at IBM Zurich - Research. His clear vision, optimism and constructive feedback have helped me to realise this ambitious research plan. He is the example of an ultra-focused scientist with a passion to develop innovative technologies to improve the livelihood of human kind. I am indebted to my supervisor Walter Karlen at ETH Zurich for his support and his wealth of wisdom conducting research. This research also could not go this far without the generous funding from the Engineering for Development from ETH Zurich, Sawiris Foundation, particularly Barbara Becker and Patricia Heuberger. Without your kind guidance, I could not be able overcome challenges after challenges. I am thankful to Walter Riess for giving me an opportunity to be part of the IBM research community. I am also very thankful to my collaborators Dr Ta Thi Tinh and Dr Duong Nguyen at the National Institute of Malariology, Parasitology and Entomology in Hanoi, Vietnam for their invaluable inputs at the early stage of my research. Sincere thanks must also be sent to Prof. Peter-Hans Beck and Sebastian Rusch at the Swiss Institute of Tropical and Public Health, who have helped and taught me many interesting lessons about malaria. The days working in Basel were so much better with the
company of Jan Warncke and the help from Francoise and Beatrice. Also, I would like to thank Professor Petra Dittrich for her interest in my research and her valuable constructive feedback as the 2nd co-examiner of my doctoral examination.

I have been blessed to work with friendly colleagues. I tremendously enjoyed the friendship and many intellectually stimulating conversations with Robert Lovchik about sciences and Switzerland. Michel Lim, Yuksel Temiz, and Onur Goekce were a pleasure to work with in the lab. I am grateful to Ute Drechsler for her technical help with the fabrication of microfluidic chips. Christophe Rossel, Nadya, Ali, Anna, Elisa, Adi and Yulieth were insightful and entertaining company during many days (and nights) working in the lab. I will always remember the warm smiles from Sylvia and Christi at the reception during many dark early winter mornings. And I also very much appreciate Manfred who was always trying to understand my requests in broken German and patiently helped to improve it.

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List of Abbreviations

ACTs – Artemisinin-based combination therapies

ASSURED – Affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable to end users

cAb – Capture antibody
dAb – Detection antibody

DNA – Deoxyribonucleic acid

FIND – Foundation for Innovative New Diagnostics

GMAP – Global Malaria Action Plan

HIV/AIDS – Human immunodeficiency virus

IGSS – Immune-gold silver staining

LAMP – Loop mediated isothermal amplification

LRS – Low-resource settings

malERA – Malaria Eradication Research Agenda

P. – Plasmodium

PATH – Program for Appropriate Technology in Health

PCR – Polymerase chain reaction

POC – Point-of-care

P. falciparum – Plasmodium falciparum

P. malariae – Plasmodium malariae

P. ovale – Plasmodium ovale
P. vivax – Plasmodium vivax

P. knowlesi – Plasmodium knowlesi

PfHRP2 – Plasmodium falciparum histidine-rich protein-2

PfpLDH – Plasmodium falciparum lactase dehydrogenase

RDTs – Rapid diagnostic tests

WHO – World Health Organization
Chapter 1

Introduction

1.1 Motivation

Although treatment for malaria is widely available, every year this disease still takes away nearly half a million lives, mostly infants under five in Africa [1]. One of the main reasons is because infections are often not detected accurately and timely [2], [3]. Most of current malaria rapid diagnostic tests (RDTs) can detect malaria infection when parasites release high concentration of antigens, for example, PfHRP2 into bloodstream [4]. When the level of PfHRP2 is low (6 ngmL\(^{-1}\)), not all commercially available antigen RDTs can identify infections [4]. This calls for a new generation of RDTs that are low cost, and capable of identifying the presence of parasites in bloodstream at onset of infections when antigen concentration is low.

The rapid growth of microfluidics technology and particularly capillary-driven microfluidics represents an emerging concept that could provide a solution for this quest. Self-driven microfluidics chips leverage the difference in pressure and hydrophilicity of microchannels to transport liquid efficiently. The aim of this thesis is to integrate a new signal amplification method, fabrication techniques and signal detection in microfluidic technology into an easy-to-use
point-of-care (POC) assay for malaria detection that replicates conventional methods but within shorter time and potentially at lower cost.

1.2 Aims

This project aims to provide a solution addressing the need for accurate diagnostic tools for early diagnosis among many current challenges of malaria elimination. The goal is to develop a robust and rapid sandwich immunodiagnostic assay on capillary-driven microfluidic platform that is capable of detecting extremely low level of malaria specific antigen. The main contributions of this thesis are:

- An examination of the current landscape and identification of gaps in malaria diagnostic technologies.
- The validation and optimisation of immune-gold silver staining (IGSS) method as an effective signal enhancement strategy for immunodiagnostic assays.
- The design capillary-driven microfluidic devices that could facilitate the silver-enhanced immunodiagnostic assays.
- The development and optimisation of on-chip signal amplification using IGSS technique for immunodiagnostic assays.
- The development of a suitable immunoassay detecting PfHRP2 with IGSS.
• An assessment of performance of capillary-driven, silver-enhanced immunodiagnostic assay detecting PfHRP2 in human serum

1.3 Outline

Chapter 2 provides an overview of the current landscape of new malaria diagnostic technologies with particular emphasis on new technologies. While symptomatic infections can be readily detected using current diagnostic methods, asymptomatic infections remain very challenging to identify. In this chapter, we look at existing in vitro diagnostic methods in malaria such as microscopy and RDTs and describe why RDTs must have high performance characteristics to be easily integrated into existing health systems for monitoring the changing malaria epidemiology. Specifically, we describe promising emerging technologies that can close the gap in sensitive diagnostics for malaria. In this review, we look at all these examples also in light of recommendations provided by Program for Appropriate Technology for Health (PATH) and Foundation for Innovative New Diagnostics (FIND) for combatting malaria, in particular for low-resource settings (LRS) where simplicity of use, appropriateness, and affordability are critical.

Then, in Chapter 3 we present a twofold strategy to improve the analytical performance of capillary-driven microfluidic chips by implementing the IGSS for signal amplification of an immunoassay, and by efficiently integrating fluorescent microbeads into microfluidic chips for immobilisation of capture antibodies (cAbs). First, we describe the assay principle, which confirms that
in presence of target analyte, metallic silver thin film is produced on the surface of the fluorescent beads and subsequently quenches the fluorescence. This attenuation in fluorescence is inversely proportional to the amount of analyte being captured by the cAbs, which are immobilised on the surface of the beads. Then we detail how the complementary bead integration is facilitated by capillarity and evaporation. We also outline the fabrication method, including bead integration and cAbs immobilisation that are amendable to mass production without compromising the bioactivity of reagents. Finally, we validate the analytical performance of the IGSS and compare to that of reference method such as ELISA on microtiter plates. The assay with silver staining on chip achieved comparable sensitivity when compared with microtiter plates assays in less than 20 min instead of hours. The limits of detection (LOD) were 17.1 ng mL\(^{-1}\) and 24.6 ng mL\(^{-1}\) respectively, suggesting excellent agreement while retaining most of the advantages of rapid tests.

In addition, we also consider the potential of such assays for POC diagnostic applications, which accepts no adverse effects due to fluctuation in temperature. We investigate the performance of the IGSS at 24 °C and 37 °C which are representative conditions found in the field. We find no significant adverse effects, if not slight improvement in terms of analytical LOD. The growth of silver over time is also characterised and in agreement with what have been reported in literature. The use of silver staining technique to amplify signal intensity also helps to mitigate the requirement for fully
transparent materials for optical measurements. Last but not least, we would like to highlight that unlike commonly used fluorescence assay, the use of fluorescent microbeads does not present limitation for POC applications since only the core of the beads is fluorescent and therefore, allow the fluorescence to be extremely stable against photobleaching.

Building on our previous work on capillary-driven microfluidic proof-of-concept, we present a strategy, which combines silver staining, a powerful method for signal amplification, with the integration of antibody receptors on fluorescent microbeads, which are self-assembled in microfluidic chips, for the precise detection of malaria antigen PfHRP2 in human serum in Chapter 4. The reagents and antigens which we use are recommended by the World Health Organization (WHO) and international agencies for developing diagnostics for malaria and evaluating tests being developed with the ones already in use. Our implementation has a very promising limit of detection (less than 6 ng mL\(^{-1}\) PfHRP2 within less than 20 min), which represents a step forward to the much needed but currently missing malaria RDT that can detect an early stage of Plasmodium falciparum (Pf) infection.

Finally, Chapter 5 presents the conclusions drawn from the results of this work in view of a commercial diagnostic test. It considers the remaining tasks needed to transform the developed prototypes into a commercial product, including possible materials to fabricate chips such as plastic and a discussion about interfacing the ‘would be’ low-cost chips with smartphone-
based fluorescent detection readers to increase its usability. The outcome of this thesis is a promising prototype that can detect extremely low concentration of PfHRP2 in human serum, hence early infection of Pf malaria, paving the way to bring the much needed RDTs closer to reality.
Chapter 2

Malaria and the detection of the ‘last’ parasite: How can technology help?

Preface

Progress in eliminating malaria has saved millions of lives, but also creates new challenges in detecting the ‘last parasite’. Effective and accurate detection of malaria infections, both in symptomatic and asymptomatic infections are needed. We present here the current progress in developing new diagnostic tools to fight malaria. We envision how an ideal rapid test for malaria elimination could look like with examples to demonstrate how innovative technologies can assist the global fight against this disease. We also identify diagnostic gaps where technology can bring an impact to the elimination campaign for malaria. This chapter is based on Malaria and the ‘last’ parasite: How can technology help, Review, Malaria Journal, 2018. https://doi.org/10.1186/s12936-018-2408-0.
2.1. The burden of malaria

Malaria is one of major health burdens with the first record of malaria fevers was dated back in the 5th century BC [5]. Today, this disease remains one of the four most life-threatening infectious diseases worldwide, together with Tuberculosis, HIV/AIDS and Hepatitis [6]. Latest data published by the WHO are staggering: more than 216 million cases in 91 countries and more than 400’000 deaths occurred globally in 2016 [1]. These figures are the same as in 2015, indicating that despite the unprecedented efforts in recent years, progress has stalled. This calls for more effective tools to reduce malaria incidences and finally to eliminate this scourge. If this historical milestone can be accomplished, it could save the global economies $2 trillion by 2040 [7].

Malaria is a common and life-threatening disease and caused by the protozoan parasite Plasmodium. There are four different species of Plasmodium: Plasmodium falciparum (P.f), Plasmodium malariae (P.malariae), Plasmodium ovale (P. ovale) and Plasmodium vivax (P. vivax) that cause malaria infections in human [5]. Additionally, humans can also be infected with Plasmodium knowlesi (P. knowlesi) species which was known to infect only animals [8]. These malaria parasites are transmitted by female Anopheles mosquitoes, which bite mainly between dusk and dawn. Once being bitten, parasites enter blood stream of human and arrive at the liver as described in Figure 1 [9].
The most severe form is caused by P. falciparum (P.f), with mild initial symptoms, which may not be accurately identified [1]. Malaria infection caused by P.f species may be fatal if treatment is delayed beyond 24 hours after the onset of clinical symptoms. Vulnerable populations include, but is not limited to infants, pregnant women, and people with weak immune systems. Malaria infections caused by other Plasmodium species result in significant morbidity, but are rarely life-threatening [1]. Infections caused by P. vivax and P. ovale are often
found in patients living in tropical areas and these parasites can remain dormant in the liver; and relapses caused by these persistent liver forms, so called hypnozoites, may appear months after exposure. Relapses are not prevented by current chemoprophylactic regimens, with the exception of primaquine [10]. *P. malariae* species causes latent blood infection which may be present for many years, but it is very rarely life-threatening [10]. *P. knowlesi* malaria is primarily a public health problem as it only affects populations living or working in forested areas in south-east Asia [1]. In recent years, sporadic cases of malaria infections found in travellers due to *P. knowlesi* have been reported [1]. In this case, monkey is the primary host and this “monkey malaria” parasite can be transmitted to human while staying in rainforests and/or their fringe areas, within the range of the natural monkey hosts and mosquito vector of this infection. The parasite has a life-cycle of 24 hours and can give rise to daily fever spikes occurring 9–12 days after infection although has no persistent liver forms and relapses do not occur [9].

The history of malaria control efforts dated back to the successful introduction of dichloro-diphenyl-tricloroethane, a vector control tool, into health systems in many countries [11]. Shortly after this success, the GMAP was initiated in 1955 by WHO, aiming to achieve full malaria control, elimination and eradication by 2015. However, GMAP was terminated in the 1970s when it became clear that the goal of malaria eradication was not achievable with
existing strategies, which underestimated the heterogeneity of malaria transmission and neglected research and development of the disease [2].

From the late 1990s, many new international programs, such as the Roll Back Malaria Partnership and the Malaria Eradication Research Agenda, were launched as the result of increased demands from endemic countries, with a consequent increase in international political and financial support from both the private and public sectors [11]. With this financial support, malaria mortality rates were reduced by 45% globally between 2000 and 2012 (Figure 2) [12].

![Figure 2: Past and projected international funding for malaria control (2000 – 2016)](chart.png)

International disbursements to malaria endemic countries increased from less than US$ 100 million (2000) to US$ 1.6 billion (2011). Increasing in international funding have slowed down in recent years, falling to an average of 4% per year (2009-2013) compared to an average increase of 43% per year (2005-2009) [12].

However, in some countries the number of malaria incidences continued to increase, either due to a real rise in incidence as a consequence of improved access to health facilities or due to the increased availability of resources for case detection during emergency response in the field (in Ethiopia the number of hospitals increased 62%, from 120 hospitals in 2005, to 195 in
Nowadays, of 97 countries with ongoing malaria transmission, 81 are focusing on disease control and 25 are at the pre-elimination stage [2]. Elimination strategies currently focus on patients with symptoms (passive case detection) (Table 1) [14]. This approach will need to shift to active case detection (detecting infections in asymptomatic individuals) and measuring transmission to ensure disease elimination [15]. Thus, now is an appropriate time to assess whether current diagnostic strategies are effective enough to achieve the ultimate goal of malaria eradication.

### 2.2. How efficient diagnostics might impact the control of malaria

#### 2.2.1 Presumptive versus parasitological diagnosis

Although malaria is entirely curable if diagnosed promptly and treated adequately [16], its symptoms are indistinguishable to other acute febrile illness and it is hence easily misdiagnosed and mistreated. Depending on the prevalence of the disease, malaria diagnosis may be symptomatic or parasitological. Presumptive treatment can achieve a sensitivity of 88% at a
specificity of 66% [17]. When the disease is highly prevalent in a region, and the clinical symptoms are fairly characteristic of malaria (high fever, fatigue, headache), syndromic management of malaria can be cost-effective and has been recommended by the WHO [18]. However, syndromic management of malaria in low transmission areas (where less than 20% of the population is infected) can accelerate drug resistance because over-diagnosis and overtreatment come at the expense of the detection and treatment of other febrile patients, leading to high mortality rates in patients mistreated for malaria [19], [20].

Treatment based on parasitological diagnosis, which is recommended by the WHO in low-transmission areas [21], has many advantages over presumptive clinical diagnosis [19], such as improved care of parasite-positive patients, detection of parasite-negative patients, and hence less misuse of antimalarial drugs, increased public confidence in healthcare systems, evaluation of treatment efficiency, improved malaria case detection, surveillance, reporting and cost benefits [13]. Therefore, while presumptive diagnosis may be sufficient for use in control settings because parasite density in patients' blood is high, parasitological diagnosis is recognized as a more effective approach in elimination settings [22].
2.2.2 Current diagnostic technologies and the challenges of detecting the ‘last’ parasite

Diagnostics is the first step towards appropriate treatment and improved clinical outcomes. Benefits of accurate and effective diagnostics include, but are not limited to more rational use of antimalarial drugs, filling in knowledge gaps and (enables) target interventions [22]. Consequently, accelerated elimination and the ability to circumvent challenges posed by insecticides and antimalarial resistances might be possible [7]. Despite these advantages of diagnostics, not all suspected cases of malaria are confirmed with diagnostic test (Figure 3) [1]. Since the development of the low cost and accurate rapid diagnostic tests (RDTs) for malaria in early 1990s, the detection of malaria cases has been increased from 74% in 2005 to 78% in 2014 [16], [23]. A significant benefit of increasing access to RDTs lies in reducing the spread of drug resistance (a reduction from 85% to 36% in the use of artemisinin-based combination therapies (ACTs) and from 27% to 37% of antibiotic prescriptions) and consequently nearly halving the rate of re-attendance at health clinics (4.9% to 2.5%) (Table 2) [24].
Table 2: Impact of diagnostic approach on medicine prescriptions (artemisinin-based combination therapies (ACT) and antibiotics), re-attendance at health facilities and over-cost per patient

Table 2 shows the outcomes of a study conducted in February to August 2005 to assess the effect of RDTs on treatment and health outcomes among 1887 febrile patients attending four primary health care facilities in Zanzibar. The study suggested that RDTs may be useful to improve the clinical management of patients presenting with fever in primary healthcare facilities, as they resulted in better health outcomes [24].

<table>
<thead>
<tr>
<th></th>
<th>Clinical diagnosis alone (n = 882)</th>
<th>Clinical diagnosis combined with RDT (n=1005)</th>
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</thead>
<tbody>
<tr>
<td>ACT prescriptions</td>
<td>85%</td>
<td>36%</td>
</tr>
<tr>
<td>Antibiotic prescriptions</td>
<td>27%</td>
<td>37%</td>
</tr>
<tr>
<td>Re-attendance at health facility</td>
<td>4.9%</td>
<td>2.5%</td>
</tr>
<tr>
<td>Cost per patient (US $)</td>
<td>2.37</td>
<td>2.47</td>
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</tbody>
</table>

Figure 3: Proportion of suspected malaria cases attending public health facilities who received a diagnostic test by WHO region, 2010 – 2016

The proportion of suspected malaria cases receiving a parasitological test among patients presenting for care in the public sector has increased in most WHO regions since 2010. The largest increase has been in the WHO African region, when diagnostic testing increased from 36% of suspected malaria cases (2010) to 87% (2016), mainly owing to an increase in the use of RDTs, which accounted for 63% of diagnostic testing among suspected cases in 2016 [1].
Despite its importance, a diagnostics-based approach has been neglected compared to the development of vaccines and treatments (Figure 4) [1].

To accelerate the elimination process, and eventually eradicate this ancient scourge, rapid tests with high sensitivity that can identify unseen reservoirs and to track the last cases are needed [3], [15]. Recently, Bell and colleagues analyzed the market for malaria diagnostics and shown that case management and surveillance are the two epidemiological settings that can most benefit from innovations in rapid diagnosis of malaria (Figure 5) [25], [26].
Unprecedented challenges for developing useful malaria diagnostics are presented: 1) in case management, there is clearly a need for field-adapted RDTs with better sensitivity than that of current malaria RDTs (≤ 100 parasites/µL). Effective and accurate diagnostic methods for malaria in pregnancy and P. vivax infections are currently absent; 2) for public surveillance, active case detection requires highly sensitive rapid tests (0 – 5 parasites/µL) that can detect asymptomatic individuals and perhaps even potentially track treatment efficacy and drug resistances. The role of innovative tools becomes crucial in the fight against malaria and WHO identifies three strategic pillars, of which accurate and effective diagnostics at the POC is the first step towards appropriate diagnosis and treatment for malaria infection [27].

Table 3 compares the performance of currently available malaria diagnostic tests for case management [28], [29], [30], [31], [32] and surveillance [31],
The landscape for malaria diagnosis can be divided into two main groups, POC methods for case management and laboratory-based methods for surveillance [37]. In case management, microscopy and RDTs are the two diagnostic methods that are used by community health workers whilst highly sensitive RDTs and molecular diagnostics polymerase chain reaction (PCR) and loop mediated isothermal amplification (LAMP) are often used by trained medical personnel. PCR and LAMP are currently the most sensitive methods, but their applications are restricted to well-equipped laboratories and trained technicians [3]. POC methods such as microscopy and RDTs, on the other hand, have been proven effective in low-resource settings (LRS) [16].
### Table 3: Characteristics of current malaria diagnostic tools used in case management and surveillance (CI: confidence interval)

<table>
<thead>
<tr>
<th></th>
<th>Limit of Detection (parasite/µL or ng/mL)</th>
<th>Sensitivity (%) (95% CI)</th>
<th>Specificity (%) (95% CI)</th>
<th>Cost ($ US/test)</th>
<th>Time</th>
<th>Other requirements</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Case management</strong></td>
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</tr>
<tr>
<td><strong>Microscopy</strong></td>
<td>Expert: 4 - 20 [28]</td>
<td>Depends on microscopist</td>
<td></td>
<td>0.12 - 0.40 [29]</td>
<td>60 min</td>
<td>Trained personnel, microscope, Giemsa stain [28]</td>
</tr>
<tr>
<td></td>
<td>Average: 50 - 200 [29]</td>
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<tr>
<td><strong>RDTs</strong></td>
<td>Existing RDTs: 100 p/µL [32]</td>
<td>&gt; 85% depending on species [29]</td>
<td>&gt; 99% [29]</td>
<td>0.55 - 1.50 [28]</td>
<td>20 min</td>
<td>Test kit, appropriate storage conditions [28]</td>
</tr>
<tr>
<td></td>
<td>Latest product: 0.8 ng/ml for PfHRP2 and 12.5 ng/mL for PfLDH [31]</td>
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<tr>
<td><strong>Surveillance</strong></td>
<td></td>
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</tr>
<tr>
<td><strong>RDTs</strong></td>
<td>Latest product: 0.8 ng/ml for PfHRP2 and 12.5 ng/mL for PfLDH [31]</td>
<td>&gt; 85% depending on species [29]</td>
<td>&gt; 99% [29]</td>
<td>No need for expensive instrument</td>
<td>0.55-1.50 [28]</td>
<td>20 min [30]</td>
</tr>
<tr>
<td></td>
<td>26 (real-time) [33]</td>
<td>100% [34]</td>
<td>&gt; 99% [33]</td>
<td>Real-time instrument: 20'000 [36]</td>
<td>&gt; 1.5-4.00 [35]</td>
<td>Standard &gt; 6h</td>
</tr>
<tr>
<td></td>
<td>0.5-5 [35]</td>
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</tr>
<tr>
<td><strong>PCR</strong></td>
<td>47 (real-time) [33]</td>
<td>83.3% [32]</td>
<td>&gt; 99% [32]</td>
<td></td>
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<tr>
<td><strong>LAMP</strong></td>
<td>≥ 1 [34]</td>
<td>97.3% [35]</td>
<td>&gt; 85% [34]</td>
<td>Conventional PCR and LAMP: ~ 5'000 [36]</td>
<td>0.40-0.70 [35]</td>
<td>60 min</td>
</tr>
</tbody>
</table>
2.2.2.1. Microscopy

Microscopy is the reference standard for visualisation of parasites in blood smears with a sensitivity under normal circumstances approximately 10-fold lower than that of molecular testing [38], [39], [40]. There are two types of tests for microscopy-based diagnosis of malaria: thick and thin blood smears [38]. The thick smear method is to detect the presence of malaria parasites in a drop of blood, and the thin smear is to distinguish the parasite species. Advantages that support the wide adoption of microscopy in the control of malaria are, but not limited to: low direct cost, high sensitivity, able to differentiate parasite species and quantitation of parasite density. The use of microscopy can also help to diagnose other diseases and enable monitoring of drug efficacy [39]. However, good-quality microscopy has been difficult to maintain, particularly in peripheral health services. One key factor that is limiting the quality of such diagnosis is the lack of effective quality assurance programs, specifically availability and skills of trained microscopists [39]. This challenge will remain dominant for the next phase of malaria management, elimination [41].
2.2.2.2. Rapid diagnostic tests

Field studies have confirmed the benefits of introducing RDTs into routine testing such as better case management, improved adherence to test results, and having more rational treatments [42], [43]. Characteristics of current malaria RDTs are summarized in Table 4.

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Easy to use</td>
<td>Deletion of the Pfhrp2 gene leads to false negative RDTs (particularly in populations in the Amazon region)</td>
</tr>
<tr>
<td>Low cost</td>
<td>Lack of adequate sensitivity for detection of infection in asymptomatic individuals and/or prozon effect</td>
</tr>
<tr>
<td>Quick result delivery time (&lt; 20 mins)</td>
<td>Lack of heat stability when being stored in endemic settings</td>
</tr>
<tr>
<td>Portable and disposable</td>
<td>Inability to differentiate non-Pf malaria</td>
</tr>
<tr>
<td>Require minimal laboratory infrastructure, power or external equipment</td>
<td>Inability to distinguish current and past infections</td>
</tr>
<tr>
<td>Quick training</td>
<td>Inability to quantify parasite density, especially for assessing severity of illness or monitoring treatment efficacy</td>
</tr>
</tbody>
</table>

Table 4: Advantages and disadvantages of current malaria RDTs

Key advantages of RDTs are the ease to use and quick result delivery time (15 to 20 minutes). Unlike PCR or microscopy, RDTs detect circulating antigen; therefore they can also be used to detect placental malaria [44]. Diagnosis of malaria in pregnancy is challenging because placental sequestration can make microscopy detection of parasites difficult. Most current malaria RDT is based on lateral flow technology [45]. The mode of action is based on lateral migration of liquid, so called immunochromatography on a porous nitrocellulose strip (Figure 6) [45].
On these RDTs, a detection antibody (dAb) which is specific to the target biomarker, is deposited onto the lower end of the nitrocellulose strip. A cAb, which is specific to the target biomarker is bound to the test line. Another antibody, specific to the dAb, is deposited on the control line. Once blood and buffer are introduced at the wells, they mixed with dAb and migrate along the porous network of the nitrocellulose materials to the lower end by capillary actions. If the target biomarker is present, it will form a complex with the dAb that is captured on the test line. In absence of the target biomarker, only the dAb gets bound to the control line. The result is available within 15 minutes if the test is performed as per instructions for use provided by the manufacturers. Although using the same technology of lateral flow
immunoassays, the performance of malaria RDTs varies greatly between brand-to-brand, and lot-to-lot, especially with specimens having low parasite density (< 200 parasites/μL) (Figure 7) [46].

![Figure 7: Malaria RDT performance against clinical sample containing P. falciparum (Pf) at different parasite densities and clean-negative samples](image)

Panel detection score in the % of malaria samples in the panel that give a positive result in two RDTs per lot at the low parasite density or by a single RDT per lot at the high parasite density. All products had a high rate of detection of Pf at high parasite density and varied performance at low parasite density [46].

Most of the evaluated malaria RDTs detect PfHRP2 or P. falciparum lactase dehydrogenase (PfpLDH). These evaluations also take into account the variations between lot-to-lot and batch-to-batch by sampling two devices from two batches. In the last round of evaluation, anomalies that interfered with result interpretation were also recorded [46]. The most common anomalies were incomplete clearing and red background, which were observed in 48% and 24% of products. The second most common anomalies were failed
migration of liquid, incomplete migration and patchy broken test lines, which occurred in 15%, 11% and 11% of the products respectively (Figure 8) [46].

In most cases, these anomalies do not invalidate the test results as reactivity in the control and test lines are still visible, but they may pose challenges to health workers interpreting the results [46].

The performance of lateral flow-based RDTs depends on two main factors: the sensitivity and specificity of antibody-antigen combinations, and the ability to facilitate reliable liquid migration on the nitrocellulose membrane. Much research has focused on new biomarker discovery and only limited attention has been paid to reduce limitations imposed by the inhomogeneous migration of liquid across porous nitrocellulose membranes [47].

Figure 9 illustrates how unstructured the flow paths could be in a nitrocellulose membrane [48]. As the migration of liquid occurs in a porous network and is not actively controlled, a number of limitations arise: large
volumes of sample, accumulation of reagents at the leading edge of the liquid flow, and increased cross-reactivity [49]. It is therefore time to consider alternative options to facilitate a more precise liquid migration, hence more accurate test results.

2.2.3. Promising and alternative technologies for malaria detection

These technologies are individually reviewed in depth elsewhere [10] and most of them rely on standard concepts using immunoassays and molecular diagnostics. Table 5 summarizes six major classes of technologies used for detecting malaria and indicates their maturity levels [10], with an emphasis on immunoassays [50], [51], molecular diagnostics [52], [53], [54], [55], [56], [57], [58], [59], [60] and the visualization of parasites [61]–[64]. Table 6 provides
Of those market-ready products, four of them are molecular diagnostics, three are immunoassays and one is based on automated microscopy. Several promising proof-of-concepts for the next generation of malaria RDTs are emerging. For example, prototypes have been built to detect the presence of hemozoin in blood sample [65]–[68]. Hemozoin crystals are produced by

Table 5: Examples of promising technologies for point-of-care diagnostics
(table based on information contained in ref [10]).
*Plasmodium* parasites as a final non-toxic compound of hemoglobin metabolism. In a specific example, a portable light meter was built to image crystalized hemozoin pigment [69]. These pigments are birefringent, so the detection of hemozoin is based on rotating a plane of polarized light through them and observing anisotropic output of the light. The minimum concentration of hemozoin that could be detected with this polarized light system was 15 pg/mL, equivalent to 30 parasites/μL of blood. Applications in the field are to be tested. Another example utilizes a portable breath analyzer: breaths of malaria-infected patients were found to contain terpenes, a family of aromatic chemicals that are produced by parasites that can further attract mosquitoes [70], [71]. A pilot study in Malawi confirmed that these aromatic compounds could be transported into the lungs and hence could be detected in the exhalation of infected patients [72]. Despite being unquestionably novel, these abovementioned methods of detection still need to prove their practicality for POC in LRS and demonstrate a clinically relevant LOD. For instance, in the breath analyzer, it would be useful to be able to convert the level of terpenes detected in breath into parasite density.
Table 6: Specifications of recently-entered market technologies for malaria diagnosis

<table>
<thead>
<tr>
<th>Technology</th>
<th>Product</th>
<th>Developer</th>
<th>Description</th>
<th>Type of detection</th>
<th>Performance</th>
<th>Turn-around time</th>
<th>Sample</th>
<th>Environmental requirements</th>
<th>Cost/test</th>
<th>Cost/Instrument</th>
<th>Power/labor/infrastructure requirements</th>
<th>Result display and storage</th>
<th>Quality control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscopy</td>
<td>Parasight</td>
<td>Sight Diagnostics Ltd, 2014</td>
<td>Automated microscopy suitable for processing of multiple malaria slides</td>
<td>Slide reading</td>
<td>Under way</td>
<td>n/a</td>
<td>Blood smear</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>RDTs</td>
<td>Flx-net</td>
<td>Flx Corporation, 2012</td>
<td>Universal RDT reader and cloud information services to improve malaria RDT quality assurance and malaria surveillance</td>
<td>Automated and customising reports</td>
<td>RDTs processing time is dependent on manufacturer's recommendations</td>
<td>Subjects to RDTs manufacturers' recommendations</td>
<td>5°C - 40°C</td>
<td>Battery powered</td>
<td>On-screen and web portal</td>
<td>CE marked</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sensitivity and specificity are functions of the RDTs being read</td>
<td>Downloaded within minutes</td>
<td>Daily quality control needed</td>
<td>5°C - 40°C</td>
<td>Basic 1 day training needed</td>
<td></td>
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<tr>
<td>Malaria RDTs</td>
<td>UM7</td>
<td>Fyodor Biotechnologies, 2015</td>
<td>A sensitive and specific lateral flow assay detecting novel Plasmodium spp. proteins shed in the urine of infected malaria patients</td>
<td>OptiDuck technology (lateral flow assay)</td>
<td>LOD 1.25 parasites/µL</td>
<td>&gt; 20 min</td>
<td>100 µL urine</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>RDTs</td>
<td></td>
<td></td>
<td>Portable, smartphone-based lateral flow immunoassay reader</td>
<td>Quantitative and qualitative</td>
<td>RDTs processing time is dependent on manufacturer's recommendations</td>
<td>Subjects to RDTs manufacturers' recommendations</td>
<td>5°C - 40°C</td>
<td>Battery powered</td>
<td>User interface of the smartphones application</td>
<td>Class I medical device</td>
<td>Basic &lt; 0.5 day training needed</td>
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<td></td>
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<td></td>
<td></td>
<td>Downloaded within seconds</td>
<td>5°C - 40°C</td>
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<tr>
<td>LAMP Malaria</td>
<td>Illumigene Malaria</td>
<td>Eiken Chemical Ltd</td>
<td>Commercial LAMP test kit containing primers and reagents to test RDTs and transmit result to a secure cloud information service</td>
<td>Isothermal DNA amplification</td>
<td>For pan-LAMP: 57.0% sensitivity 94.2% specificity</td>
<td>Stable for 12 months at &lt; 30°C</td>
<td>1500</td>
<td>Electricity (battery-powered possible)</td>
<td>Turbidimeter and software</td>
<td>Positive and negative controls included</td>
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<tr>
<td></td>
<td></td>
<td>and FIND, 2012</td>
<td></td>
<td>For Py-LAMP: 53.0% sensitivity 85.5% specificity</td>
<td>Fluorescence of visual detection</td>
<td>60 min</td>
<td>30 - 60 µL blood</td>
<td>$15</td>
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<td>ISO 9608:1993</td>
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<td></td>
<td></td>
<td>Sensitivity 90%</td>
<td>&lt; 50 min</td>
<td>Human whole blood</td>
<td>n/a</td>
<td>n/a</td>
<td>Does not require specialised laboratory equipment</td>
<td>n/a</td>
<td>CE marked</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>Specificity 90.3%</td>
<td>Stabile for 12 months at 2°C - 30°C</td>
<td>$150</td>
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<td>1 - 2 days training required</td>
<td>$18500</td>
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* G6PD point-of-care tests are not included due to lack of information for popular products. CareStart G6PD RDT (AccessBio) and POC G6PD (PATH) are working on promising products.
* recently-entered market means product passes the regulatory and policy stage.
2.3. Specifications for a new generation of malaria RDTs

The introduction of RDTs into national malaria programs has transformed the case management in many countries [24]. However, limitations of current health systems might hinder the effect of such testing program; for instance, uncertainty over deployment of RDTs over presumptive treatment does not change the prescribed practice of doctors in many countries as expected [19]. Slow regulatory approval and lack of standard procurement processes, low inventories of RDTs supplies, lack of in-field quality assurance for RDTs are just a few weaknesses of the current health systems in developing countries that are slowing down the elimination process of malaria. Nevertheless, these problems call for a better harmonized health management system where accurate diagnostics can be a starting point [73]. The merging of rapid detection technologies with diagnostic target concentration has led to the vision of an ideal RDT diagnostic platform for malaria elimination: an integrated platform that will require little user input, the result will be displayed in an easy-to-interpret manner, possibly with handheld devices and supply management software that allow data transmission for surveillance purposes (Figure 10).
Different settings require different target product profiles (TPP) [3]. Unlike previous malaria control campaigns, the key characteristics of malaria elimination efforts are to interrupt endemic transmission and to prevent its re-establishment [74]. PATH and FIND are pioneering the development and validation of sensitive rapid tests for mass screening in LRS. They also proposed a TPP for malaria RDTs in elimination settings, stating specific requirements for the ideal rapid tests according to concept of Affordable, Sensitive, Specific, User-friendly, Equipment-free and Deliverable (ASSURED) [75]. The desired LOD is 5 parasites/µL or less, which is equivalent to a concentration range of 6 to 12 ng/mL PfHRP2 [75]. For RDT developers it is important to note the caveat of the prozone phenomenon that...
might prevent detection of high parasite density [76]. Poor specificity could lead to over-treatment, thus depreciation of the intended value of RDTs (from public health perspectives); therefore the required specificity for effective malaria diagnosis is at least 97% or ideally 99% [75].

Additional requirements for ideal RDTs are suitability and appropriateness for LRS where most malaria cases occur. To make an impact simplicity and affordability are of utmost importance. Simplicity means, the system should be equipment-free and should require very little resources [77]. A simple and automated test could obviate false results caused by user-errors [78]. Affordability is difficult to measure and depends on the cost-benefit equation of a specific situation. Also, tests should be designed to minimize impact of inappropriate storage conditions (2 °C – 40 °C) on reagent stability and usability of the devices [79].

2.4. Microfluidic technology for malaria POC testing

Microfluidics enable the miniaturization and simplification of complicated analytical processes while consuming less reagents, minimizing waste, and requiring less supporting instrumentation [80]. This stems out from the predictable behavior of liquids at the microscale where flow is typically laminar. At the microscale, minute amounts of liquids can be manipulated using microstructures such as microvalves, micromixers or micropumps to facilitate immunodiagnostic assays on chip [81]. Low volumes of reagents,
fast reaction times, compact and portable platforms, are just a few advantages that make microfluidics technology attractive for POC applications [82], [83].

Figure 11 shows several examples demonstrating the archetype of microfluidic-based diagnostics for POC applications, which is an integrated system composed of a disposable unit (where analysis takes place) and a signal acquisition and processing module to process the results [84], [57], [85].

![Figure 11: Examples of microfluidic-based diagnostics for low resource settings](image)

(a) a microfluidics-based point-of-care dongle capable of detecting HIV and syphilis simultaneously within 20 minutes [84], (b) a lab-on-a-disk diagnostic platform that can detect multiple biomarkers in one run [57], and (c) an easy-to-use DNA detector that can detect Ebola infection at the point-of-care [85].

Currently, microfluidic-based diagnostic devices can be divided into two categories: “traditional” microfluidics and paper-based microfluidics [86], [87]. Research on traditional microfluidics often focuses on miniaturizing
conventional techniques. For example, a collection of passive and active mixing elements were designed to facilitate mixing processes on chips [88]. Recent work in developing microfluidic-based diagnostic devices has focused on integrating all necessary elements into stand-alone platforms [89], [90] because such integrated systems can operate without bulky accessories and do not require water, buffer, or a constant supply of electricity [81], [91], [92], [93], [94] (Figure 12).

There are many ways to control liquid flows on microfluidic platforms, for instance, acoustic forces, mechanical forces, magnetic forces, as well as capillary and centrifugal forces [95]–[99]. To satisfy the stringent requirements for LRS, devices based on capillary and centrifugal forces have shown promising results. Table 7 presents some examples of microfluidic-based systems that have been designed to detect PfHRP2 and PfLDH antigens or genetic materials from the parasites using on-chip molecular testing [100], [101], [102] cell deformation mechanism [103], [104], [105], electrical [106], optical [107], [69], [90], [108], and magnetic detections [65], [109] amongst others.
a) Evolution timeline of microfluidics technology

- 1950's: First R&D of liquid analysis at high precision
- 1980's: Microfluidic liquid manipulation research
- 1990's: Early microfluidic life science companies founded
- 2000's: Microfluidic diagnostic devices successfully commercialised
- 2010's: Increased development of diagnostic products

b) Suitability evaluation of microfluidics-based diagnostic platforms for malaria detection

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<tr>
<td>Peripheral blood smear</td>
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<td>Rapid Diagnostic Tests (RDTs)</td>
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<td>Nucleic Acid Amplification (PCR)</td>
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<td>Microfluidic diagnostic devices</td>
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Figure 12: Microfluidics-based diagnostics for malaria detection

(a) adapted from IBM 2010 [81], (b) the format is adapted from Tay et al (2016) [92], *adapted from the WHO ASSURED (Affordable, Sensitive, Specific, User-friendly, Robust & Rapid, and Deliverable), ** adapted from Drain et al (2014) [93] and *** adapted from Gascoyne et al (2004) [94].
Table 7: Performance of proof-of-concept platforms based on microfluidics for malaria detection

<table>
<thead>
<tr>
<th>Application</th>
<th>Concept / Detection principle</th>
<th>Biomarker / Target</th>
<th>Performance</th>
<th>Time (min)</th>
<th>Ref</th>
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<tr>
<td>Molecular analysis</td>
<td>Paper-based LAMP</td>
<td><em>P. falciparum</em></td>
<td>61% 98%</td>
<td>45 min</td>
<td>[100]</td>
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<td></td>
<td></td>
<td><em>P. vivax</em></td>
<td>81% 98%</td>
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<td><em>P. pana</em></td>
<td>&gt; 80% &gt; 98%</td>
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<td>Continuous flow PCR</td>
<td><em>P. falciparum</em></td>
<td>2 p/µL 97.40% 93.80% n/a</td>
<td>2.5 h</td>
<td>[102]</td>
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<td>&lt; 1 p/µL n/a n/a</td>
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<td>Cell deformation mechanism</td>
<td>Inertial focusing</td>
<td><em>P. falciparum</em></td>
<td>2 - 10 p/µL n/a n/a</td>
<td>400 µL/min</td>
<td>[103]</td>
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<td>Inertial microfluidics</td>
<td><em>P. falciparum</em></td>
<td>2 cells/min n/a</td>
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<td>[104]</td>
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<td><em>iRBCs</em></td>
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<td>Non-inertial lift effect</td>
<td><em>P. falciparum</em></td>
<td>Enrichment factor of 4.3 n/a</td>
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<td>[105]</td>
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<td></td>
<td></td>
<td><em>ring stage</em></td>
<td>Throughput 12'000 cells/h</td>
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<td>Electrical detection</td>
<td>Electrical conductivity of iRBCs is significantly higher than healthy RBCs</td>
<td><em>P. falciparum</em></td>
<td>n/a</td>
<td></td>
<td>[106]</td>
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<tr>
<td>Optical detection</td>
<td>Optofluidic flow analyser that can measure the optical absorption of RBCs in <em>P. falciparum</em>-infected blood sample</td>
<td><em>P. falciparum</em></td>
<td>1712 RBCs/s 2.96% parasite density n/a</td>
<td>3 min</td>
<td>[107]</td>
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<td>Naked-eye screening of in-meso detection of hemozoin crystals based on birefringence</td>
<td>Hemozoin crystals produced by <em>P. falciparum</em></td>
<td>n/a</td>
<td>~12 min</td>
<td>[69]</td>
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<td>Visual detection of colored assay spot on a disposable microfluidic card based on a flow-through membrane immunoassay</td>
<td><em>Malaria pHRP2</em></td>
<td>10 - 20 ng/mL n/a</td>
<td>1 - 5 min</td>
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<td>Paper-based cartridge containing detection area for both thin and thick smears</td>
<td><em>P. falciparum</em></td>
<td>100 p/µL n/a</td>
<td>30 min</td>
<td>[108]</td>
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<td>Magnetic detection</td>
<td>Cell enrichment microfluidics combined with magnetic relaxometry detection</td>
<td><em>P. falciparum</em></td>
<td>5% parasite density n/a</td>
<td>15 min</td>
<td>[65]</td>
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<td>Detection of hemozoin in iRBCs by magnetic resonance relaxometry</td>
<td>Hemozoin in iRBCs in <em>P. falciparum</em> infections</td>
<td>&lt; 10 p/µL n/a</td>
<td>few mins</td>
<td>[109]</td>
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RBC: red blood cell, iRBC: infected red blood cell
2.4.1 Immunodiagnosticso n microfluidic platforms for malaria detection

Standard protocols to perform immunodiagnosticso n microfabricated platforms require sample pre-concentration, flow control and detection of biomarkers (analytes and/or parasites). These multi-step protocols can benefit greatly from miniaturization, and in fact, microfluidic-based immunoassays have demonstrated their potential for reliable and accurate performance [110], [111]. Figure 13 presents some examples to illustrate how microfluidics technology can be used to detect malaria by different methods of detection such as molecular testing [100], size-based cell sorting [103], electrical differentiation of healthy and infected red blood cells [106], optical detection of antigen [90] and magnetic detection of hemozoin [109].

2.4.1.1 Sample pre-concentration

Low antigen concentration is a common problem in diagnostic immunoassays and malaria antigen detection is not an exception. To overcome this challenge, several prototypes of analyte concentrator have been developed to enrich biomarkers hence improve LOD. To illustrate how analyte enrichment of analyte prior to analysis can improve sensitivity of ELISA, Cheow et al. reported a prototype that can enhance the LOD of prostate-specific-antigen assay up to 1.85 pg/mL [112]. The significant enhancement of 100-fold was achieved by trapping the charged fluorescent product of standard ELISA
Figure 13: Examples of microfluidic prototypes for malaria diagnosis using different methods for malaria diagnosis. (a) a paper-based multiplexed LAMP detection of malaria in blood [100], (b) a label-free inertial microfluidic system for cell enrichment and purification of malaria parasites from blood [103], (c) a dielectrophoresis stretching of healthy and infected red blood cells [106], (d) detection of multiple biomarkers from whole blood [90], and (e) micromagnetic resonance relaxometry for rapid label-free malaria diagnosis [109].
(analyte-bound enzyme complex) using a multiplex electrokinetic preconcentration technique without modifying the immunobinding process. Blood is the most common type of specimen for POC testing. However, the cellular components in whole blood often cause non-specific background. To address this problem, a continuous microfluidic device was developed to filter the cells, making plasma available for on-chip analysis [113]. Healthy and P.f-infected red blood cells exhibit different ionic permeability of their plasma membrane, with infected cells being more permeable. Therefore, when healthy and infected cells are suspended in a low conductivity medium, infected cells lose internal ions and acquire a different dielectrophoretic mobility than healthy ones [114]. Several groups have developed microfluidic chips using dielectrophoresis and variants of it to separate cells successfully leading to promising prototypes for detecting infected red blood cells thus malaria infections [115], [116], [117]. Some examples are provided in Figure 14 to illustrate the feasibility of on-chip cell-sorting approach [90], [103], [112], [118], [119], [113].
Figure 14: Sample preconcentration in microfluidic platforms

(a) a pneumatic actuation system to control liquid flow with integrated reagents [90], (b) inertial microfluidic platform to sort cells based on different morphologies of healthy and infected red blood cells sizes [103], (c) an electrokinetic concentrator to enrich analyte to improve sensitivity of [112], (d) a centrifugal platform to extract DNA from whole [118], (e) a device with porous silica membrane to preconcentrate proteins [119], (f) a continuous blood plasma separator based on Zeifach-Fung effect [113].
2.4.1.2 Flow control

Controlling flow on microfabricated devices often introduces a great degree of complexity, for examples, pneumatic actuation valves [120], capillary forces [121], soft valves [122], vacuum-driven forces [89], [123] and bubble-mixing approach [124] (Figure 15).

Figure 15: Passive fluid control in microfluidic platform

a) a pneumatic valve system to enable serial dilution and analyse multiple antigens [120], (b) libraries of capillary valves [121], (c) soft valves to control flow [122], (d) a stand-alone, self-powered integrated microfluidic blood analysis system [89], (e) a micro-chaotic mixer [123], (f) bubble-mixer [124].
For example, a combination of screws, pneumatic and solenoid valves was integrated into a microfluidic platform to actuate flow and control chemical gradients in microchannels [125]. This design might be suitable for laboratory-based tests, but may not lead to robust systems for LRS. Nonetheless, the uses of centrifugation and capillary forces to transport liquids are excellent examples of stand-alone systems [126], [127]. Extensive reviews discussing how to engineer flow path in microscale using capillary and centrifugal forces for POC applications exist [81], [128].

2.4.1.3 Detection
Sensitive detection remains one of the biggest hurdles for clinical diagnosis at the onset of infection. The bottleneck is the limited amount of detectable analytes in a very limited volume of sample. One strategy is to amplify the signal, then convert it into quantitative measurements such as electrical and/or optical signals [111]. The detection strategy is therefore critical for the overall design and fabrication of a device. Optical detection is considered as the ideal read-out for POC applications of microfluidics owing to the simple design and potentially low cost [129], [130]. There are five main categories of optical detection based on the type of generated optical signals: fluorescence, luminescence, absorbance, surface plasmon resonance, and surface-enhanced Raman scattering [131]–[135]. Detailed discussions about detection strategies for microfluidics systems also exist in the literature with
some highlighted examples such as labeled optical detection [136], electrical detection [137] and colorimetric detection [138] provided in Figure 16.

![Figure 16: Examples of POC detection strategies for quantitative measurement of malaria biomarkers](image)

(a) a miniaturised immunogold-based assay developed to detect malaria antigen using smartphone [136], (b) an integrated electrochemical detection system on chip [137], (c) detection and quantitation of malaria antigen on a foldable card [138].

### 2.4.2 Molecular testing on microfluidic platforms for malaria detection

At the moment, molecular testing (PCR and LAMP) is the most sensitive technique for identification of asymptomatic individuals with a LOD up to 3.6
× 10^{-4} parasite/μL by amplifying the presence of ribosomal RNA (of *Plasmodium* parasites) [139]. However, current applications of PCR and LAMP are still restricted to well-equipped laboratories and thus not suitable for LRS [140].

Miniaturized PCR and/or LAMP is desirable, but developing such devices is a more challenging task than that for biomarkers detection for three reasons: 1) sample pre-treatment is essential for extracting DNA of parasites for downstream analysis, 2) the critical signal amplification step highly depends on temperature control, and 3) robust, low cost, and portable detection techniques are required for remote settings (Figure 17) [141].

*Figure 17: Typical process for a biochemical assay in a microfluidic device* adapted from [141].
2.4.2.1 Sample pre-treatment

The PCR/LAMP process requires isolation of genetic materials from infected cells, probably pre-concentration, as well as signal amplification and analysis. All steps need to be integrated seamlessly in a closed process to overcome time consuming laboratory-like processing steps. Earlier studies have demonstrated successful prototypes that could sequentially perform cell isolation and lysis for messenger RNA purification [142], such as dielectrophoresis [115], cell sorting prior to extraction [103], and combination of lateral flow and chromatography [143] (Figure 18). On this device, a unique valving system was designed to facilitate liquid migration and analysis. Microfluidics with “macrofluidics” can also be combined to precisely reconstitute reagents, and automated filling liquids for multiplex PCR technique. A successful story is the Cepheid GeneXpert instrument, where all steps from sample preparation, nucleic acid extraction, to thermal cycling for amplification and eventually detection can be integrated into one platform [144]. A review of microfluidic-based DNA analysis systems is available here [145].
Figure 18: Example of cell isolation and nucleic acid extraction using combination of microfluidics with different methods

(a) dielectrophoresis field-flow-fractionation to sort iRBCs and extract DNA from parasites [115], (b) using inertia forces to isolate Plasmodium [103], and (c) a combination of lateral flow assay and chitosan-based chromatography to extract DNA from specimen [143].
2.4.2.2 Heating systems

The major challenge of miniaturizing bench-top PCR instruments is the requirement of numerous heating cycles for thermal reactions, some examples are provided in Figure 19 [101], [102], [146], [147]. To overcome this challenge, micromixers and microchambers were designed to allow thermal reactions to take place rapidly [148]. To speed up DNA amplification by improving thermal transfer through interfaces, microfluidic elements such as mixers, heaters and temperature controlling units were integrated into glass and silicon substrates [149]. Another strategy to enable different heating regions using continuous flow was investigated using a Peltier element to regulate the temperature for thermal cycling [101]. On this platform, as few as to 2 P. falciparum parasites/µL could be detected. This device offered a simplified sample processing step using desiccated hydrogel, reagents and a camera to detect amplicons. A field-testing of this prototype in Uganda with 188 patients achieved 97.4% sensitivity and 93.8% specificity.

One of the most promising development for stand-alone integrated systems for DNA analysis perhaps was an elegant combination of an exothermic reaction with phase change materials to regulate the heat for thermal cycling [150]. In this prototype, downstream processes such as purification and concentration of sample were integrated seamlessly into the same platform.

Recent work reported by Juul et al. challenged the need of thermal cycling for PCR-like systems by proposing an endogenous enzyme activity detection
called rolling-circle enhanced enzyme activity to quantify as little as 1 P. \textit{falciparum} parasite/μL [102].

The principle of this method is based on using rolling-circle-amplification (RCA) technique to convert a circular DNA template into a $10^3$ tandem repeat rolling-circle product. In this system, RCA substrates can be processed by the DNA-cleaving enzyme topoisomerase I from \textit{Plasmodium} parasites, which produces many DNA circles leading to enhanced signal. RCA products can have sizes reaching micrometers, which enable visualization at single molecular level.
Figure 19: Signal amplification approaches for microfluidic PCR

(a) an integrated microfluidic elements incorporated to perform electrophoresis on chip [101], (b) a continuous flow PCR with different heating regions [102], (c) a droplet-based microfluidic chip to perform rolling-circle enhanced enzyme activity [146], (d) a method to control temperature for LAMP application [147].
2.5. Conclusion

Accurate and effective diagnosis is the first step to further pursue efforts to eliminate and reduce the global burden of malaria by 90% in 2030. Current diagnostic methods can detect malaria symptomatic infections, but often miss out asymptomatic cases. The rise in proportion of asymptomatic infections in low transmission areas calls for a new generation of rapid diagnostic tests that can detect the hidden parasite reservoir. Technology is advanced nowadays to (at least theoretically) track down the last parasite carriers. While malaria case management has improved, other causes of fever need to be detected and treated accordingly. Therefore, the ideal RDT should come in as a complete package with ultra-high sensitivity and specificity, meet the ASSURED standards for LRS, and also provide additional diagnostic capabilities. Microfluidic devices connected to phone-based readouts offer a unique opportunity to not only reduce the burden of infectious diseases such as malaria, but also could provide tools for monitoring epidemics and elimination progress on very large scales.
Preface

POC diagnostics are critically needed for the detection of infectious diseases, particularly in remote settings where accurate and appropriate diagnosis can save lives. However, it is difficult to implement immunoassays, and specifically immunoassays relying on signal amplification using silver staining, into POC diagnostic devices. Effective immobilization of antibodies in such devices is another challenge. Here, we present strategies for immobilizing cAbs in capillary-driven microfluidic chips and implementing a gold-catalyzed silver staining reaction. We illustrate these strategies using a species/anti-species immunoassay and the capillary assembly of fluorescent microbeads functionalized with cAbs in "bead lanes", which are engraved in microfluidic chips. The microfluidic chips are fabricated in silicon (Si) and sealed with a dry film resist. Rabbit IgG antibodies in samples are captured on the beads and bound by dAbs conjugated to gold nanoparticles (NPs). The gold NPs catalyze the formation of a metallic film of silver, which attenuates fluorescence from the beads in an analyte-concentration dependent manner.
The performance of these immunoassays was found comparable to that of assays performed in 96 well microtiter plates using "classical" ELISA. The proof-of-concept method developed here can detect 24.6 ng mL\(^{-1}\) of rabbit IgG antibodies in PBS within 20 minutes, in comparison to 17.1 ng mL\(^{-1}\) of the same antibodies using a ~140-min-long ELISA protocol. Furthermore, the concept presented here is flexible and necessitate volumes of samples and reagents in the range of just a few microliters. This chapter is based on *A bead-based immunogold-silver staining assay on capillary-driven microfluidics*, Research Article, Biomedical Microdevices, 2018. https://doi.org/10.1007/s10544-018-0284-6.
3.1. Introduction

Healthcare facilities in the developing world consist not only of centralised laboratories in well-equipped hospitals in cities, but also of primary healthcare centres with limited infrastructure in peripheral regions. In such settings, POC diagnostics are critical for guiding appropriate treatments for infectious diseases such as malaria, in which the delay between diagnosis and treatment can be life threatening [151]. Developments of innovative technologies has transformed POC diagnostics to bring healthcare services closer to patients [152]. There are many benefits that well reflect this transformation, for example the increasing number of rapid antigen detecting tests, or POC immunodiagnostic assays for malaria and HIV [10], [153].

There are two main diagnostic platforms that are being used for POC applications: conventional lateral flow assay technology and emerging microfluidics-based technologies [154], [155]. While the lateral flow technology has a long history with a number of successful stories in commercialising diagnostic products, there are only very few microfluidics-based tests available in the market [156].

Sensitive detection and simplicity of use are prerequisites for POC diagnostic assays [82]. In other words, compelling performance and affordability are current challenges for commercialization of microfluidics-based diagnostic chips [156]. Fluorescence, luminescence and absorbance are three optical
detection methods that have been implemented for signal transduction of the immunobinding reactions in low cost, microfluidics-based POC diagnostics [129], [157], [158]. Fluorescence-based assays offer high sensitivity and selectivity [159]. Such immunoassays rely on fluorophores that are linked to dAb and that can emit a fluorescence signal upon excitation using a specific wavelength. The intensity of the fluorescence signal is proportional to the amount of antigens selectively captured on a surface. Organic fluorescent dyes and quantum dots are commonly used as labels. For example, an ultrasensitive fluorescence immunoassay was developed to simultaneously detect two cancer biomarkers, carcinoma embryonic antigen and α-fetoprotein, in serum using CdTe/Cds quantum dots as fluorescent probes [160]. The LOD of this fluorescence immunoassay on the microfluidic chips was 250 femtomolar, which is 3 orders of magnitude better than that of assays using conventional fluorescence probes. Issues that are hindering further progress on fluorescence-based POC diagnostics are the (photo)stability of fluorophores and the cost, complexity and fragility of fluorescence readers. Luminescence is a method used in variants of standard immunosorbent assays. In luminescence immunoassays, an enzyme conjugated to the dAb converts a substrate into a product that emits light [132]. Unlike fluorescence, this method does not require an optical excitation source, therefore luminescence detection is simpler than that of fluorescence in terms of optics. In a recent example showing the implementation of this technique in a portable and microfluidics-based diagnostic platform, a
chemiluminescent assay detecting C-reactive protein (CRP), a biomarker that indicates inflammation in the body when its concentration exceeds 5 µg mL\(^{-1}\), was developed [161]. In this prototype, the LOD for CRP reached 4.27 ng mL\(^{-1}\), which is comparable to conventional chemiluminescent immunoassays in laboratories. Furthermore, the compact design of this prototype with all-integrated reagents and pre-programmed on-chip mechanical valves for controlling the steps of the assay showed the technical feasibility of implementing a luminescence assay into a POC platform. Nevertheless, the stability and cost of the enzyme used for such assays need to be addressed. Hydrogen peroxide or chemicals that are needed for chemiluminescence are unstable at ambient conditions and require storage at low temperatures, which is also cumbersome for the development of fully integrated POC diagnostic devices.

Perhaps the most popular detection method used in commercially-available diagnostic immunoassays is the one based on absorbance detection. Absorbance-based assays rely on the conversion of a substrate into a strongly coloured product by an enzyme, which is linked to a dAb [162]. Although being slightly less sensitive than fluorescence- and luminescence-based immunoassays, these assays offer a reasonable performance and can involve relatively low cost reagents and chemicals [163], [164]. To improve the performance of assays using absorbance measurements, metal NPs such as gold NPs are used to intensify colorimetric signals of assays [165].
Typically, the size of gold NPs varies from 10 to 50 nm [166]. Larger gold NPs might create steric hindrance for ligand-receptor interactions and smaller gold NPs can be challenging to visualize [167]. Holgate et al. proposed to use IGSS to solve this problem by depositing silver on the surface of gold NPs to enhance the signal intensity after the ligand-receptor binding has taken place [168]. To investigate the suitability of IGSS for diagnostic assays, a lateral flow assay for influenza was engineered, detecting hemagglutinin of H5-type of influenza viruses [169]. LOD of the silver amplified assay was decreased 500 times compared to that of the assay without silver amplification, and this LOD was 10 times lower than that of commercial influenza rapid diagnostic tests. Similarly, the IGSS method was used to detect HIV and rubella infections in blood samples and the LODs were comparable to the reference methods [170], [171]. The specificity of both assays using IGSS was also higher and interferences due to cellular components of whole blood specimens were less prominent than with other commonly used diagnostic methods.

In this chapter, we present a capillary-driven microfluidic chip for IGSS with cAbs for analytes located on the surface of microbeads. We specifically developed an assay using species and anti-species polyclonal antibodies to demonstrate how such assay can be implemented. There are three key features in this approach. First, the beads are self-assembled from a bead suspension into specific structures of the chip and the chip is sealed by
lamination with a dry film resist (DFR) layer at low temperature. This makes the integration of beads and biological receptors into the microfluidic chip simple, fast and versatile. Second, the core of the beads is fluorescent and the growth of a metallic silver layer during the silver staining step efficiently attenuates/quenches this fluorescence, which also eliminates the need for fully transparent microfluidic devices for measuring absorption of light by the formed silver film. The fluorescence signals are extremely strong when no analyte is present in the sample and fluorescent beads are highly stable against photobleaching. Third, the microfluidic chip is designed to accommodate sub-microliters of solutions, which are sequentially pipetted for the IGSS assay. All handling steps for the assay take as little as 20 min and there is no need for active microfluidic elements to perform the assay.

3.2. Materials and methods

3.2.1 Antibodies and reagents

All reagents were purchased from Sigma-Aldrich unless otherwise stated. Water was purified using a Simplicity 185 system (Millipore, Billerica, MA). Phosphate buffered saline (PBS) solution was prepared by dissolving commercially available PBS tablets. A solution of 1% w/v bovine serum albumin (BSA) was prepared in PBS. PBS with Tween 20 (PBST) was prepared by adding Tween 20 to PBS to a resulting concentration of 1%. 3,3'-
5,5′-tetramethylbenzidine in water (BM Blue POD substrate) was used as a substrate for enzyme-based immunoassays. A 1:1 v/v mixture of silver A and silver B from SE 100-1 KT silver enhancer kit was prepared prior to the signal amplification step. Streptavidin-coated 96-well microtiter plates and blocker BSA solution were from Life Technologies. Fluorescent streptavidin PMMA beads (PolyAn GmbH, Red 25, 5.9 µm diameter) were used as carriers to integrate the cAb in microfluidic chips. Biotinylated anti-rabbit IgGs was used as cAb. Donkey anti-rabbit IgGs conjugated with 6 nm gold nanoparticles (abcam) and donkey anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (abcam) were used as dAbs. Rabbit IgGs and mouse IgGs were used for positive and negative control experiments for immunoassays on 96-well microtiter plates.

3.2.2 Fabrication materials

Si wafer (Si-Mat, Kaufering, Germany) and SU-8 photoresist (SU-8 3010, MicroChem Corp.) were used to fabricate microfluidic chips. Acetone and isopropyl alcohol were used to clean the microfluidic chips after dicing. DFR (DF-1050, Engineered Materials Systems, Inc., USA) was used to seal the chip.

3.2.3 Protocols for assays

To validate the reagents and characterize the assay protocol, the IGSS assay was first performed using standard streptavidin-coated 96-well microtiter
plates. Between each step, the plates were washed three times using a plate washer (Tecan). First, 50 µL of a biotinylated anti-rabbit IgG solution (10 µg mL⁻¹) was added into each well and incubated for 30 min at room temperature (RT). Then solutions of rabbit IgG (positive control) and mouse IgG (negative control), were added into each well using a serial dilution factor of 2 with the starting concentration 4 µg mL⁻¹ in the first wells (50 µL/well). The incubation was 30 min at RT. For silver staining, a solution of gold conjugate was diluted 1:2000 (v/v) in 1% BSA in PBST and added into each well (50 µL/well). Incubation of the analyte with dAb-conjugated gold was 15 min at RT. Solutions of silver A and silver B were mixed (1:1, v/v) and added immediately into each well (50 µL/well). The silver development step was 20 min, at RT and in the dark (plates covered with an aluminium foil). For standard enzymatic assays, a solution of HRP conjugate was diluted 1:10'000 (v/v) in 1% BSA in PBST and added into each well (50 µL/well). This incubation was 60 min at RT. Solutions containing POD blue were added (50 µL/well) and the enzymatic reaction was allowed to proceed for 20 minutes. To measure the end-point absorbance, a plate reader (Sunrise, Tecan) was used at a wavelength of 570 nm and the absorbance measurements were taken at RT.

For immunoassays performed on microfluidic chips, aliquots of solutions were sequentially added to the loading pads of the chip. Fluorescence images of the beads integrated into the microfluidic chip before and after the silver staining step were taken using a fluorescence microscope (Nikon Eclipse 90i,
Japan) equipped with a 20× objective and a Texas Red fluorescence filter. Excitation of fluorophores was done using an LED Lumencor lamp (software SOLA S2 Controller). Images were taken using a black and white CCD camera (DS-1 QM, Nikon) and an acquisition time of 400 ms and using a ND16 filter. The software Fiji (ImageJ) was used to analyse the fluorescence images. Fluorescence images for regions of interest (ROI) comprising the beads before and after silver staining were acquired and the mean fluorescence intensity for each experiment was obtained by subtracting the background signal around an ROI to the mean signal value in the ROI.

3.3. Results and discussion

3.3.1 Assay and detection principles

The strategy to implement a silver staining technique for microfluidics-based immunoassays is outlined in Figure 20. Two steps are performed off-chip, Figure 20A. First, fluorescent microbeads, which are functionalized with streptavidin, are incubated with biotinylated cAb (1:5 v/v). These beads are then integrated into specific microstructures (“bead lanes”) so that the cAbs are localized in a well-defined area of the chip. The sample containing the analytes (antigens) is then mixed with a solution of dAb conjugated to gold nanoparticles for 1 min (1:1 v/v). The assay takes place by having this solution passing over the integrated beads, leading to the capture of antigen-
dAb complexes by cAb on the beads, Figure 20B. Gold nanoparticles on the dAb then catalyze the deposition of silver from a staining solution. The formation of this silver metallic film attenuates the emitted fluorescent signal of the beads, which provides optical signal readout. The type and volume of solutions sequentially added to a loading pad of the microfluidic chip are listed.
in Figure 20C. Each added solution starts to flow in the microfluidic chip owing to capillary action. When the solution has flown for the desired time, the excess solution left in the loading pad is removed using a cleanroom tissue and the next solution is added. The first added liquid is a 1% solution of BSA in PBS and it is allowed to flow for 1.5 min (i.e. ~53 nL based on optically monitoring the advancement of the solution in the chip) to block surfaces and prevent non-specific adsorption of analytes and dAbs up to the receptor areas. The assay proceeds with the addition of the pre-mixed antigen-dAb solution (1:1 v/v), which is allowed to flow for 5 min (~175 nL). The antigen-dAb complex is captured by the cAb present on the beads during this step, Figure 20B. Then, deionised water is added for a 4-min rinsing step (~140 nL) before adding a silver staining solution, which is allowed to flow for 7 min (~246 nL). In presence of antigens, a film of metallic silver forms on the surface of beads. During this silver staining step, the microfluidic chip is covered with an aluminium foil to avoid non-specific, light-catalysed reduction of silver. The addition of deionised water displaces the silver staining solution where beads are located and stops the formation of the silver film so that the fluorescence emitted from beads can be measured.

3.3.2 Chip design and bead integration

The microfluidic chip for implementing the IGSS assay is shown in Figure 21. The chip is approximately 1 cm² in size and contains six types of microfluidic elements: anti-wetting structures, 2 loading pads, a flow resistor, 2 bead lanes,
a capillary pump, and an air vent, Figure 21A. All structures are 15 µm deep and defined in a layer of SU-8 with the exception of bead lanes, which are etched in the Si layer itself. These bead lanes accommodate beads functionalized with cAb, Figure 21B. Each lane can hold approximately 200 beads and the beads are solely present in the bead lanes after their integration to the chip as seen in the SEM image of Figure 21B.

Once a liquid is pipetted onto a loading pad, it follows the microfluidic elements along the flow path owing to capillary action. The flow resistor has the function of slowing down the flow so that sufficient time can be obtained
for the binding of analytes to cAbs on the bead or for the silver staining reaction. Up to 5 µL of liquid can be added to a loading pad and semi-circular ridges around pads act as anti-wetting structures and prevent spreading of the added liquid to the adjacent loading pad or edges of the chip due to liquid pinning. Depending on the desired volume of liquid that needs to enter the flow path, excess of liquid in a loading pad can be readily removed using a cleanroom wiping paper. Volumes of liquids added to the chip are estimated based on the observed flow rates and filling state of the capillary pump. The IGSS assay demonstrated here only requires ~700 nL of sample and reagents. The assay conditions can be adapted by varying the dimensions of the flow resistor and capillary pump, which impact both the flow resistance of the flow path and the capillary pressure along it.

The fabrication steps for making the microfluidic chips are shown in Figure 22. The fabrication uses standard clean room processes and only two photolithography masks. A low-cost single side polished Si wafer is used as the substrate. First, the bead lane structures are etched anisotropically to a depth of 15 µm using deep reactive-ion etching (DRIE) and a 1.2 µm positive-tone photoresist as the masking layer. This etching step takes only 3 minutes per wafer and feature sizes as small as 2 µm can be patterned without any optimization. Based on preliminary experiments for integrating beads using different channel depths for the bead lanes, a minimum channel depth of 10 µm creates enough capillary pressure to completely wet the bead lane when
the top surface is still open. Following the Si etching step, a 15 µm thick SU-8 layer is patterned for the microfluidic structures using the recipe provided by the supplier. The presence of the etched bead lanes does not adversely affect the uniformity of the spin-coated SU-8 layer because the bead lanes occupy less than 2% of the total chip area. The wafer is then diced to half of its thickness using a semi-automated dicing tool to define individual chips. The
partially diced wafer is immersed into acetone then rinsed in isopropyl alcohol to clean the surface for the bead integration step.

The bead integration step is initially facilitated by capillary actions followed by evaporation of carrier solution, Figure 22B. 0.5 µL bead solution in 1% BSA in PBST (0.01%) (0.5 % w/v) is introduced at the bead loading pad when the chip surface is still open. Bead solution flows orthogonal to the main channel and only beads are trapped at the bead lane. More bead solution can be loaded if needed until the bead lane is completely filled with beads. Lateral spreading of the excess liquid in the main channel is minimized owing to the lower capillary pressure in the main channel compared to the bead lanes. Because the beads self-assembly process happens without using any external energy, many bead lanes can be filled with beads in a short time by serial deposition of the carrier solution. This makes the technique compatible with mass production using automated pin spotters. Following the integration of the beads, the chip surface needs to be sealed to create a strong capillary pressure of samples required for the assay and to minimize evaporation. Many sealing techniques are available for microfluidic applications, such as adhesive or thermal bonding. However, these techniques are typically not compatible with integrated reagents because of the use of solvents and/or high temperatures. Here, the sealing of the chip is performed by gently laminating a 50-µm-thick DFR at 45 °C. This low temperature and fast (<10 s) sealing process ensures proteins coated on the bead surface are minimally
affected, Figure 22C. In addition, it is not required to structure the DFR to pattern the openings for the loading pads. A straight cut aligned to the loading pads is sufficient to hold the pipetted liquid owing to the anti-wetting structures. Because all channel walls are inherently hydrophilic, no additional surface treatment is needed for the capillary flow. Finally, ready-to-use chips can be singulated by manual breaking through the partially diced regions, Figure 22D [172]. This step also breaks the DFR, making the whole process compatible with wafer-level reagent integration and sealing. For a standard clean room using 200 mm Si wafers, this process would yield more than 300 chips per wafer.

3.3.3 Assay implementation validation

ELISAs are very well-established assays and therefore can be used as a gold standard to evaluate microfluidics-based immunodiagnostic assays. We compared the sensitivity between IGSS and ELISA carried out on microtiter plates using a bench-top plate reader for absorbance measurements. The titration data for both types of assays are fitted using a 4-parameter logistic regression (4-PL) (Figure 23).

The LOD for these assays is defined as the smallest concentration of analyte for which the signal is above the mean signal + 2 standard deviations (95% confidence interval) of samples not containing analytes. For an immunoassay detecting rabbit IgG, the LOD of the IGSS was 17.1 ng mL⁻¹, while the LOD of
the ELISA was 103.2 ng mL\(^{-1}\). This result indicates that the performance of IGSS is slightly superior to bench-top ELISAs.

With the aim of implementing the IGSS in immunodiagnostic assays for low resource settings, the effect of temperature on the assay performance were tested by detecting rabbit IgGs at ambient temperature (24 °C) and elevated temperature (37 °C) (Figure 24). These two conditions represent the temperature range often found in standard laboratories and in the field. The titration data for both types of assays are fitted using a 4-PL (Figure 24A). The LOD were estimated to be 17.1 ng mL\(^{-1}\) and 6.1 ng mL\(^{-1}\) for assays performed at 24 °C and 37 °C respectively. This result suggests that an elevated temperature does not have detrimental effect on the performance of the IGSS but rather slightly improves its sensitivity (Figure 24B). To

Figure 23: Comparative absorbance measurement of ELISA and the IGSS immunocapture of rabbit IgGs. Standard deviations correspond to triplicate of experiments and shown as error bars. Dotted lines represent the cut-off values of each method (based on the average values of negative samples and two times the standard deviations).
characterize the growth of the silver film over time, the IGSS assay was performed using a sample containing a rabbit IgG at a concentration of 4 µg mL\(^{-1}\) on microtiter plates and the silver staining process was stopped using three minute intervals. Absorbance measurements taken using a bench-top plate reader are presented in Figure 24B. The growth of the silver film can be divided into three phases: first, the silver growth exhibits a slow initial phase for approximately 9 minutes, then the silver staining evolves rapidly between 9 to 21 minutes. During this time, the thickness of the silver film is proportional to the duration of the silver reduction reaction. After 21 minutes, the silver staining process reaches plateau, which is typical of electroless plating reactions and in agreement with previously reported data [173].

Figure 24: Kinetics and temperature characteristics of the silver staining reaction in an IGSS assay performed using 96-well microtiter plates

For all experiments, the dilution of gold-conjugated dAb was 1:2000. (A) Comparison of the silver staining step performed at 24.3 °C and 37 °C after 20 min and using optical absorption measurement. (B) The concentration of rabbit IgG in 1% BSA in PBS was 4 µg mL\(^{-1}\). Silver staining was stopped at three minute intervals by addition of deionized water and the optical absorbance of the corresponding silver layer was measured using an UV/Vis plate reader. Standard deviations of triplicate experiments are shown as error bars.
Next we wanted to investigate if the silver staining method for signal amplification of immune-capture reactions is compatible with capillary-driven microfluidic chips. To this end, we used microfluidic chips with integrated fluorescent microbeads functionalized with anti-rabbit antibodies, samples and solutions of reagents as described earlier. Specifically, a serial dilution of rabbit IgG in 1% BSA in PBS was prepared and such solutions were pipetted on the first loading pad of microfluidic chips and allowed to flow into the chip for 5 min. Each chip was only used once and a fluorescence micrograph around the bead lanes was acquired 7 min after the silver staining solution had been added to the chip. The dependence of the fluorescence attenuation from the beads (microfluidics-based assay) or of the light absorption (microwell-based assay) with the concentration of rabbit IgG in the samples are displayed in Figure 25 together with some representative images of the silver films and bead lanes. These experiments were performed at RT (24°C) and in both cases the signal correlates with the concentration of analyte in the sample leading to an increased absorption of light or emitted fluorescence as the concentration of analyte increases. The obtained signals are fitted using a4-PL and the LOD of the microwell- and microfluidic chip-based assays were found to be 17.1 ng mL⁻¹ and 24.6 ng mL⁻¹ respectively. Such LODs are very similar and in fact represent an excellent sensitivity considering that polyclonal antibodies were used for this proof-of-concept and that the assay on the microfluidic chip necessitated only 20 min and few microliters of solutions.
Figure 25: Performance comparison of the IGSS assays

(A) capillary-driven microfluidic chips and (B) 96-well microtiter plates. For all experiments, the dilution of gold-conjugated dAb was 1:2000. (A) 1. Fluorescence micrographs correspond to the attenuation of fluorescence from beads by the stained silver layer after 7 min loading silver mixture. 2. Fluorescence profile of the bead-based IGSS assay on capillary-driven microfluidic chips. The fluorescence images were taken after 7 min flowing of silver mixture on the chip. Standard deviations of triplicates in a single experiment are shown as error bars. Dotted lines represent the cut-off value for this IGSS assay at 24.6 ng mL⁻¹. (B) 1. Photographs of micro-wells correspond to silver staining layers obtained with four different concentrations of rabbit IgG after 20 min. 2. Optical absorbance measurements of the silver layer obtained from IGSS assays performed on 96-well microtiter plates. The absorbance measurements were taken after 20 min incubating with silver mixture then rinsing. Standard deviations of 9 repeats are shown as error bars. Dotted lines represent the cut-off value for this IGSS assay at 17.1 ng mL⁻¹.
3.4. Conclusion

A microfluidic chip with successful implementation of silver staining and complementary integration of microbeads was developed. The use of fluorescent beads for microfluidics-based immunodiagnostic assays is not a limitation for point-of-care applications because many fluorescence devices have been engineered and fluorescence readers have been developed for various point-of-care applications. The assay with silver staining on chip achieved comparable sensitivity when compared with microtiter plates assays in less than 20 min. Moreover, the implementation of immunogold silver staining in microfluidics system eliminates the need for fully transparent devices when absorption of light is measured, and allows a simple in-plane optical detection system. Effect of temperatures on performance of assays was investigated, presenting no adverse effect for the silver staining immunoassays. While shelf life of reagents and their stability was not investigated in this work, this should not cause any issues since antibodies, commercially-available silver staining solutions and reagents are stable and commonly used for both research and in vitro diagnostic applications. The proof-of-concept prototype developed in this work achieves similar performance to that of lab-based immunoassays while retaining most of the advantages of rapid tests. The time taken to carry out an assay is only 20 min and there is no need for active microfluidic elements. Once the liquid is introduced, the flow is self-driven. The chips are designed to be compact and
simple to use. Furthermore, the potential for mass production is considered by using dry film resist for easy fabrication, and by utilizing a design on Si that can easily be transferred to available mass production technologies. Possibly, this system can be fully integrated with automatic microfluidic delivery of buffers and reagents. Overall, we demonstrated strategies with which microfluidic chips and microbeads can be combined with silver staining to achieve a performance comparable to a reference lab-based method.
Chapter 4

Towards a rapid diagnostic test for malaria elimination using immunogold-silver staining assay on capillary-driven microfluidics

Preface

Accurate and affordable RDTs are indispensable but often lacking for many infectious diseases. Specifically, there is a lack of highly sensitive malaria RDTs that can detect low antigen concentration at the onset of infection. Here, we present a strategy to improve the sensitivity of malaria RDTs by using capillary-driven microfluidic chips and combining sandwich immunoassays with electroless silver staining. We used 5 µm fluorescent beads functionalized with cAbs. These beads are self-assembled by capillary action in recessed "bead lanes", which cross the main flow path of chips microfabricated in Si and SU-8. The binding of analytes to dAbs and secondary antibodies (2nd Abs) conjugated to gold NPs allows the formation of a silver film on the beads, which masks the fluorescent core of the bead inversely proportional to the concentration of antigen in a sample. We illustrate this method using the recombinant malaria antigen rPfHRP2 spiked in human serum. This antigen was a recombinant HRP2 protein expressed in Escherichia coli, which is also the standard reference material. The LOD of
our immunoassay was found to be less than 6 ng mL\textsuperscript{-1} of rPfHRP2 within 20 minutes, which is approaching the desired sensitivity needed in the TPP for malaria elimination settings. The concept presented here is flexible and may also be utilized for implementing fluorescence immunoassays for the parallel detection of biomarkers on capillary-driven microfluidic chips. This chapter is based on \textit{Toward a rapid diagnostic test for malaria elimination using immunogold-silver staining assay on capillary-driven microfluidics}, Research Article, Biosensors & Actuators B: Chemical (submitted).
4.1. Introduction

Today many cases of infectious diseases remain untreated even though the diseases are curable. One of the main reasons for this situation is that many patients do not have access to diagnostic services. Furthermore, late diagnosis of infectious diseases such as malaria increases the risk of its spreading and makes treatment more difficult [174]. To address this challenge, the WHO published in April 2018 its first Essential Diagnostics List, a catalogue of essential tests needed to diagnose global priority diseases such as malaria, HIV and tuberculosis, as well as most common conditions such as diabetes [175]. For test developers, the release of this list indicates the importance of designing tests such as malaria RDTs that are affordable, safe, and of high quality.

As the declining trend in global malaria burden has stalled, efforts to eliminate this scourge require disruptive innovations to obtain more powerful tools [3]. Accurate and effective malaria RDTs remain a key intervention leading to better treatment and achieving the ambitious goal of eliminating malaria by 2030 [27], [176]. Most current malaria RDTs are based on a lateral flow assay format, which is cheap to produce and easy to use. However, the major disadvantage of this type of test is the inability to detect low antigen concentrations or low parasite densities at early stages of infection [176]. A TPP was proposed by FIND and PATH to define the desired LOD at 6 ng mL$^{-1}$ PfhHRP2 for the ideal malaria RDTs detecting HRP2 in elimination settings.
There are only few malaria tests that can meet this requirement at present [46], [177], [31].

There are several examples of microfluidic chips that have been developed with promising analytical performance. We reviewed the current landscape of malaria RDTs and how recent advancement in technology can help in detecting the last few parasites in malaria elimination settings [178]. Like current malaria RDTs, many of them use a nitrocellulose membrane, or so-called paper-based microfluidics, to facilitate the detection of malaria. Yager et al. demonstrated a lateral flow immunoassay incorporated on a paper-patterned ‘card’. On this card, all reagents were stored in dry state [179]. By folding the card, a sequential chemical analysis could be triggered, resulting in the detection of malaria PfHRP2 [90]. Cooper et al. developed a paper-based LAMP device that is capable of detecting and differentiating Plasmodium falciparum, Plasmodium vivax and being pan-specific for the other Plasmodium species directly from finger-pricked samples within 45 minutes [100]. It certainly would be beneficial to further develop these technologies using less reagents and improving sensitivities and precision.

Furthermore, it is challenging for test developers to evaluate the performance of RDT prototypes due to a lack of standardised reference materials. To avoid this caveat, it is important to have access to established reference materials at early stages of test development. In a joint project of the WHO Global Malaria Programme, FIND and CDC, the performance of commercially available
malaria RDTs has been systematically evaluated and compared using high quality of panels of recombinant antigens [180].

To address the above-mentioned shortcomings of current malaria RDTs, we developed an assay for detecting malaria PfHRP2 achieving clinically relevant LOD. This builds on our previous work on the IGSS assays and capillary-driven microfluidic chips where species and anti-species Abs were used to perform a sandwich immunoassay on beads. These beads are fluorescent, functionalized with cAbs, and self-assembled by capillary action in microstructures of the chips. The accumulation of antigens and dAbs conjugated to gold NPs on the functionalized beads results in the electroless deposition of a silver film, which leads to a corresponding attenuation of the fluorescence signal from the core of beads [181]. Here, we functionalized the fluorescent microbeads with cAbs directed against PfHRP2 (denoted as α-PfHRP2 or cAb). In presence of PfHRP2, a complex between this antigen and dAbs conjugated with gold NPs is formed and captured on the surface of the microbeads. Then, gold NPs catalyse the formation of a silver film on the microbeads with a continuous flow of reagents for silver staining. The attenuation of fluorescence emitted from the core of the beads strongly correlates to the concentration of rPfHRP2 spiked in human serum samples, leading to a highly sensitive detection of malaria PfHRP2 infection.
4.2 Materials and Methods

4.2.1 Antibodies and reagents

All reagents were purchased from Sigma-Aldrich unless otherwise stated. Deionised water was obtained from a Simplicity 185 system (Millipore, Billerica, MA). PBS was prepared by dissolving one PBS tablet in 200 mL of deionised water. Solutions of 1% and 5% w/v BSA in PBS were prepared by dissolving BSA in PBS solution. Solutions of PBST 0.1% and 0.01% were prepared by adding appropriate volume of Tween 20 to PBS solutions. A monoclonal mouse α-PfHRP2 IgG 2b (G18K) (100 µg mL$^{-1}$, ThermoFisher Scientific) was used as cAb. A monoclonal mouse α-PfHRP2 IgG 2a (H36F) (1.0 µg mL$^{-1}$, ThermoFisher Scientific) was used as dAb. A monoclonal rabbit α-mouse IgG 2a (1.0 µg mL$^{-1}$, Abcam) was used as 2ndAb. Polyclonal donkey anti-rabbit IgGs conjugated with 6 nm gold nanoparticles and polyclonal donkey anti-rabbit IgGs conjugated with enzyme horse radish peroxide (HRP) were from Abcam. 3,3’-5,5’-tetramethylbenzidine in water (BM Blue POD substrate) was used as a substrate for enzyme-based immunoassays. A 1:1 v/v mixture of silver A and Silver B from SE 100-1 KT silver enhancer kit was prepared prior to the signal amplification step. Transparent Costar 96-well microtiter plates were from Sigma-Aldrich.

Fluorescent streptavidin PMMA beads (PolyAn GmbH, Red 25, 5.9 µm diameter) were used as substrates to integrate cAb into microfluidic chips. An
antibody concentration kit (Abcam) was used to enrich the concentration of cAbs 10 times for the biotinylation step. A biotin fast conjugation kit (type B) (Abcam) was used to biotinylate cAbs. Recombinant HRP2 protein (rPfHRP2) expressed in *Escherichia coli* (Microcoat Biotechnologie GmbH, Germany) based on the HRP2 sequence of the W2 P. *falciparum* strain was selected as target analyte. A serial dilution of target analyte was prepared in 1% BSA solutions in PBS and human serum (Biowest) for assays on microtiter plates and in microfluidic chips, respectively. Prior to use, human serum was filtered using 0.2 µm diameter blood filter (Millipore).

4.2.2 Fabrication of microfluidic chips and integration of beads

Microfluidic chips were fabricated on 4-inch Si wafers (Si-Mat, Kaufering, Germany). Wafer layout comprising 28 chips and 2 layers was written to 5 × 5 inch² glass/Cr photomasks (Nanofilm, USA) using a direct laser writing tool (DWL 2000, Heilderberg Instruments). A mask aligner (MA6, SÜSS MicroTec AG, Germany) with ani-line filter (365 nm) was used for the photolithography steps. Structures used to integrate beads (i.e. bead lanes) were etched using a DRIE tool (AMS 200, Alcatel, France) while masking the wafer surface with AZ®6612 positive-tone photoresist (MicroChemicals GmbH, Germany). Following the removal of the photoresist in an O₂ plasma ash (600 W, 600 sccm, 5 min), microfluidic structures were fabricated using photolithographic patterning of a 15-µm-thick SU-8 (SU-8 3010, MicroChem Corporation) using the protocol provided by the supplier. The fabricated wafer was partially
diced using a dicing tool after protecting the wafer surface with AZ®6612 photoresist. The wafer was cleaned using acetone and isopropyl alcohol, and dried. Prior to the bead integration, streptavidin-coated, fluorescent beads were incubated with cAbs and rinsed with 1% BSA in PBST (0.01%). Then, about 100 nL of bead suspension (0.5% w/v) in 1% BSA in PBST (0.01%) was introduced at the bead loading pad when the chip surface was still open (not laminated). Beads functionalized with receptors were carried by capillary flow orthogonally to the main channel and get trapped at the bead lane after drying of the solution. More bead suspension can be loaded if needed until the bead lane is completely filled with beads. Following the integration of beads, diced microfluidic chips were sealed by lamination of 50-µm-thick dry film resist (DFR) (DF-1050, Engineered Materials Systems, Inc., USA) using a hotplate at 45 °C and a plastic spatula.

4.2.3 Protocols for assays

For immunoassays performed on microtiter plates, transparent Costar 96-well microtiter plates were used. Between each step, the plates were washed three times using a plate washer (Sunrise, Tecan). First, 50 µL of a cAb solution (1.0 µg mL⁻¹) was added into each well and incubated for 60 min at room temperature. Then solutions of 5% BSA in PBS was added to block nonspecific binding (50 µL/well) for 30 min. Next, serial dilution of GST-PfHRP2-W2 in 1% BSA in PBS was added into each well (starting concentration of analyte was 50 µg mL⁻¹, 50 µL/well). Analyte incubation was
allowed to last 60 min at room temperature. Additions of dAb (1.0 µg mL\(^{-1}\)) and 2\(^{nd}\)Ab (1.0 µg mL\(^{-1}\)) were followed and each step was incubated for 30 min. For silver staining, a solution of gold conjugate was diluted 1:2000 (v/v) in 1% BSA in PBST and added into each well (50 µL/well). Incubation of the analyte with dAb-2\(^{nd}\)Ab-conjugated gold was 30 min at room temperature. Silver mixture consisted of silver solution A and silver solution B, which were mixed (1:1, v/v) and added immediately into each well (50 µL/well). The silver development step was 20 min, at room temperature and in the dark (plates covered with an aluminium foil). For standard enzymatic assays, a solution of HRP conjugate was diluted 1:10000 (v/v) in 1% BSA in PBST and added into each well (50 µL/well). This incubation was 30 min at room temperature. Solutions containing blue POD substrate were added (50 µL/well) and the enzymatic reaction was allowed to proceed for 20 minutes. To measure the end-point absorbance, a plate reader was used with a 570-nm wavelength. Standard deviations of three repeats in a single experiment were shown as error bars.

For immunoassays performed on microfluidic chips, the cAb was enriched 10-fold in concentration from 100 µg mL\(^{-1}\) to 1.0 mg mL\(^{-1}\), then biotinylated. Protocols for the enrichment and biotinylation of cAbs are standard procedures provided by Abcam. A sample containing the analyte in human serum, dAb, 2\(^{nd}\)Ab, gold conjugated Ab and an aliquot of 0.01% Tween 20 in deionised water were sequentially added to the first loading pad of the microfluidic chip. The silver staining solution and then 0.01% Tween 20 in
deionised water were then added to the second loading pad of the chip. Images of fluorescent beads integrated in bead lanes were taken using a fluorescence microscope (Nikon Eclipse 90i, Japan) equipped with a 20× objective and a Texas Red fluorescence filter cube. A LED Lumencor lamp (software SOLA S2 Controller) was used to excite fluorophores. Images were taken using a monochrome CCD camera (DS-1 QM, Nikon) and an acquisition time of 400 ms with ND16 filter. Fluorescence images were analysed using software Fiji (ImageJ). Regions of interest (ROI) comprising the beads before and after silver staining were selected from acquired fluorescence images and the mean fluorescence intensity for each experiment was obtained by subtracting the background signal around an ROI from the mean signal value in the ROI. Standard deviations of six repeats in a single experiment were shown as error bars. For all assays, titration data were fitted using a 4-parameter logistic regression (4-PL) model. The LOD was defined as the lowest concentration of PfHRP2 for which the signal is 3 × standard deviation (99% confidence interval) above the mean signal of samples not containing PfHRP2.
4.3 Results and discussion

4.3.1 Validation of IGSS for malaria PfHRP2 detection using a commercial RDT and a microtiter plate

As a first step, we used a commercial RDT to provide a reference for the sensitivity achieved using low cost tests. We chose the commercial malaria RDT Paramax-3 (Zephyr Biomedicals) to detect the biomarker rPfHRP2, because this RDT had passed round 2 testing of the WHO/FIND/CDC product evaluation with 93% PDS, which is a criterion taken into account for analytical performance but also to test inter- and intra-lot variations [182]. A serial dilution of rPfHRP2 in 1% BSA in PBS with was prepared in accordance to the test protocol used in the round 2 testing with three rPfHRP2 concentrations of 1880 ng mL$^{-1}$, 188 ng mL$^{-1}$ and 18.8 ng mL$^{-1}$. The test procedure was followed as defined by the manufacturer and we found that the Paramax-3 detected a concentration of 188 ng mL$^{-1}$ PfHRP2 and higher, but could not detect a PfHRP2 concentration of 18.8 ng mL$^{-1}$ (Figure 26A).

Next, we developed an assay protocol for the IGSS. IGSS necessitates a dAb conjugated to NPs that can catalyse the electroless deposition of silver, and ideally such an antibody should be directed against PfHRP2, monoclonal (monoclonal antibodies lead to more specific assays), and commercially available. We were not able to identify such a dAb and therefore decided to use additional species/anti-species antibodies to circumvent this issue. We
use a monoclonal dAb α-PfHRP2 from mice and a rabbit α-mouse 2ndAb. The gold NPs are then functionalized with a donkey α-rabbit Ab (Figure 26).

The last antibody (α-rabbit Ab) could also be conjugated to HRP to perform an enzymatic assay using a colorimetric readout. Interestingly, the use of 2ndAbs has been suggested in the literature as a mean of improving the sensitivity of assays [183].

Figure 26: Performance of malaria rPIHRP2 assays using various formats

(A) an example of a commercial malaria RDT Paramax-3 (Zephyr Biomedicals) used to detect the malarial rPIHRP2 antigen at three concentrations. Absorbance measurements of (B) an IGSS assay and (C) an ELISA on microtiter plates for various concentrations of rPIHRP2 in PBS. Insets in (B) and (C) represent the assay architecture for each method. Standard deviations correspond to triplicate of experiments and shown as error bars. Blue lines represent the signal generated from samples containing PIHRP2 and red lines represent the signal generated from samples without PIHRP2. Dotted lines represent the limit of detection of each method (based on the average values of samples without PIHRP2 plus three times standard deviations).
We evaluated the sensitivity of the IGSS assay using the above-mentioned antibodies and antigens (Figure 26B) and compared this sensitivity to that of a well-established reference method, namely an ELISA involving an enzymatic-based signal (Figure 26C). Both assays were performed on microtiter plates using a bench-top plate reader for colorimetric absorbance measurements. For the immunoassay detecting PfHRP2 on microtiter plates, the LOD of the IGSS assay was 105 ng mL⁻¹, while the LOD of the ELISA was 49 ng mL⁻¹. These results indicate that the IGSS assay is a little less sensitive than ELISA and approximately as sensitive as the commercial malaria RDT.

4.3.2. IGSS assay protocol developed for the microfluidic chip

We transposed the IGSS assay to an assay on a capillary-driven microfluidic chip [98] for the detection of malaria antigen rPfHRP2 in human serum specimen. For this we start with two off-chip preparation steps (Figure 27A). The first step is to coat the fluorescent beads with cAbs prior to their integration to the microfluidic chip. The second step is to incubate rPfHRP2 in human serum with dAbs, 2ndAbs and gold conjugated antibodies for 5 min. The on chip assay starts with the addition of 2 µL of the pre-incubated solution from above onto a loading pad of the capillary-driven microfluidic chip to let the solution pass over the integrated beads for the capture of the rPfHRP2-complex for 5 min (Figure 27B).
Next, a rinsing solution is added to the chip to remove unbound antibody complexes to avoid non-specific silver staining. Three microliters of silver staining solution are then added to the chip to catalyse the reduction of silver ions using the gold NPs conjugated to the 2nd Abs. The formation of the silver
film is then stopped after 7 min by adding 2 µL of deionised water containing 0.01% of Tween 20. The rinsing solution is allowed to flow in the chip for another 2 min before acquiring the fluorescence signal using a fluorescent microscope. These steps are summarized in Figure 27C and in practical terms, once being added into a loading pad, each solution flows along the microfluidic path due to capillary action. When the solution has flown for the desired time, the excess solution left in the loading pad is removed using a cleanroom tissue and the next solution is added. A capillary pump at the end of the flow path ensures that capillary action is maintained each time a solution is added to a loading pad and the characteristics of the capillary pump were set to support a flow rate of ~40 nL min⁻¹ [184]. To avoid non-specific deposition of silver due to light, the microfluidic chip is covered with an aluminium foil during the silver staining step. The formation of this silver film attenuates the emitted fluorescence signal of the beads, which allows for optical signal readout by measuring the fluorescence emitted from beads before and after the silver staining step.
4.3.3 Fabrication of the microfluidic chip and integration of beads

The microfluidic chips are patterned on a Si wafer using standard photolithography and engraving techniques (Figure 28).

Flow paths for the assay are defined in SU-8 (lateral walls), SiO₂ (native oxide of silicon substrate, bottom wall), and a dry film resist (DFR) (top wall). The flow path for integrating the beads functionalized with the receptors is defined in the silicon substrate using photolithography and dry etching (Figure 28A). 100 nL of a bead suspension is applied to a loading pad servicing a microchannel crossing the main flow path of the chip and ending with narrow outlets. Beads accumulate in this recessed microchannel in a few seconds by capillary assembly and the solution evaporates in ~20 s (Figure 28B).

Anti-wetting structures patterned around the bead integration zone prevents undesired spreading of the bead solution. This self-assembly process
conveniently localizes receptors at a precise location of the chip without the need for specific surface chemistry or patterning of the surfaces forming the flow path. No post-processing or rinsing step is needed but instead the chip can be sealed, stored or directly used for an assay.

Lamination uses a DFR with low autofluorescence and is done at 45 °C (Figure 28C) [172]. This sealing technique is chosen for three main reasons. First, this low temperature and fast (< 10 s) lamination does not affect the biological activity of cAbs immobilised on the integrated beads. Second, there is no need to pattern the DFR for the openings of the loading pad and finally this lamination does not require any solvents. The chip then can be singulated by hands and ready for use (Figure 28D) [172].

4.3.4 Chip design

The microfluidic chip is designed as an ensemble of connected microelements in which multiple liquids are displaced by capillary actions (Figure 29). The chip is ~1 cm² and comprises five key microfluidic elements: anti-wetting structures, two loading pads, two bead lanes where cAbs are immobilised and the signal is measured, a capillary pump for drawing the liquid at a programmed flow rate, and an air vent. The flow path is 15 µm deep, 100 µm wide and defined by lateral walls in SU-8.
These small dimensions of the microstructures lead to short paths for diffusion of analytes and reagents during an assay and efficient rinsing steps owing to the laminar flow of liquids filling the chip. Furthermore, semi-circular ridges around loading pads act as anti-wetting structures, keep liquids pipetted in loading pads and prevent liquid from overflowing loading pad. Volumes of liquids flowed in the chip can be estimated by optical monitoring of the advancement of the solution in the chip. The assay conditions can be easily adapted by either varying the dimensions of the capillary pump, or the addition of flow resistors, or even electrogates for stop-and-go control of liquids filling the chips [185].

Figure 29: Image of a microfabricated Si microfluidic chip for IGSS immunoassays for detecting PfHRP2.

The flow path starts with 2 parallel loading pads that are surrounded with anti-wetting structures, a connection channel linking the loading pads to "bead lanes", a capillary pump, and air vent. All microfluidic structures are formed in a 15-μm-high photoresist layer excepted for the bead lanes, which are etched in the Si layer. The chip is 15-mm-long and 6.5-mm-wide (97.5 mm²). The insets are SEM micrographs of a bead lane (15-μm-deep, etched in Si and 20-μm-wide) before and after the bead integration.
The flow path directs a sample over bead lanes, which are 15-µm-deep and 20-µm-wide structures in Si that hold ~200 beads with a diameter of 5.9 µm, (Figure 29, inset). The SU-8 lateral walls forming the flow path are patterned so as to be distributed at least 50 µm away from the bead lanes to avoid interferences with the autofluorescence of SU-8 when acquiring the fluorescence signals from the beads before and after the silver staining step. The discontinuity in the flow path does not compromise flow in the microfluidic chip because the chip is sealed and only a few nanolitres of solution can spread laterally toward the areas where the bead suspension is loaded or drawn.

4.3.5 IGSS performance

We investigated the performance of the IGSS specifically for a malaria rPfHRP2 assay in human sera. To this end, we used microfluidic chips having fluorescent microbeads coated with α-PfHRP2 antibodies, rPfHRP2 in human sera, solutions and reagents as described earlier. A serial dilution of rPfHRP2 was prepared in human serum. Each of the dilution was incubated with a mouse monoclonal α-PfHRP2 IgG 2a, monoclonal rabbit α-mouse IgG 2a, and a polyclonal gold conjugated donkey α- rabbit IgG for 5 min before use. Each chip was only used once and fluorescence micrographs of the bead lanes were acquired before and 7 min after adding a silver staining solution to the first loading pad. These experiments were performed at room temperature
(24 °C) and the measured optical signal did correlate with the concentration of therPfHRP2 antigen in the sample (Figure 30).

The obtained data are fitted using a 4-PL model and presented together with some representative fluorescent micrographs of the bead lanes after silver staining in Figure 30.

Figure 30: Detection of rPfHRP2 using the IGSS assay and capillary-driven microfluidic chips.

(A) Relative change of the fluorescence measured from beads before and after the silver staining step as a function of the concentration of rPfHRP2 spiked in human serum (blue line and data points) and for non-spiked human serum (red lines). The dotted red line represents the cut off value for this IGSS assay at less than 6.0 ng mL\(^{-1}\) rPfHRP2 in human serum. (B) Fluorescence micrographs showing the attenuation of fluorescence from beads by the stained silver layer.

The LOD of the IGSS malaria assay performed on microfluidic chips was found to be below 6 ng mL\(^{-1}\) PfHRP2. To put the results achieved from this study into perspective, we summarize the LODs of the IGSS assay on capillary-driven microfluidic chips, ELISA and IGSS assay on microtiter plates, and the LODs of some commercial malaria RDTs [31], together with the desired LOD for the ideal malaria RDTs for elimination settings [75], (Table 8).
These figures suggest an excellent sensitivity of our assay given that the assay only needed 20 min and few microliters of sample and reagents.

<table>
<thead>
<tr>
<th>LOD (ng mL(^{-1}) PfHRP2)</th>
<th>IGSS microfluidic chip</th>
<th>Microtiter plates</th>
<th>Commercial RDTs</th>
<th>Ideal malaria RDT for elimination settings</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 6.0</td>
<td>IGSS(^a)</td>
<td>ELISA(^a)</td>
<td>Paramax-3(^a)</td>
<td>best-in-class RDT(^a)</td>
</tr>
<tr>
<td>Time (min)</td>
<td>20</td>
<td>140</td>
<td>20</td>
<td>20</td>
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<tr>
<td></td>
<td></td>
<td>10</td>
<td>10 to 20</td>
<td></td>
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</table>

Table 8: Comparison of the LOD of various methods used for detecting PfHRP2
\(^{a}\) results obtained in this work, \(^{b}\) [31] and \(^{c}\) [75]).

4.4 Conclusions

A microfluidic chip prototype for point-of-care (POC) diagnostics was developed based on a unique combination of a fluorescence assay, self-assembled functionalized beads in a capillary-driven microfluidic chip and electroless silver staining technique for the detection of PfHRP2 with a promising LOD for early diagnosis of malaria. Since only the core of the beads is fluorescent, photobleaching and stability issues, which are often found in many fluorescent immunoassays are mitigated. The use of fluorescent beads does not present a limitation for POC applications as many handheld peripheral fluorescence readers have been developed for use at the point of need. The attenuation of fluorescence intensity emitted from the core of the beads due to formation of a silver film alleviates the need for fully transparent devices. The assay achieved a sensitivity below 6 ng mL\(^{-1}\) of PfHRP2, which is in line with the desired sensitivity specified in the TPP for
malaria elimination settings. Although not being investigated in this study, the fluctuation in temperature should not cause any adverse effects on silver staining. The short turnover time of 20 min supports the use of this technology in the field where health workers often have to perform many tests in a day, or when patients have to receive treatment within the same visit. The microfluidic prototype reported here relies on capillary forces to facilitate sequential chemical analysis without any active elements. This makes the microfluidic chips compact and easy to use while retaining all advantages of rapid tests. Potentially, automatic reagent and buffer delivery elements can be incorporated so that this device could become a ‘one-step’ test that has been envisioned for the ideal malaria RDT. This work might be extended to the fabrication of disposable polymeric chips using state-of-the-art roll-to-roll, injection molding or hot-embossing techniques. In light of the TPP for malaria elimination settings, the prototype reported here represents a step forward and more generally may incentivize the adoption of fluorescent bead-based immunoassays using capillary-driven microfluidics for POC diagnostics applications.
5.1 Conclusions

A microfluidic chip prototype for POC diagnostics was developed based on a unique combination of a fluorescence assay, self-assembled functionalized beads in a capillary-driven microfluidic chip and electroless silver staining technique for the detection of PfHRP2 with a promising LOD for early diagnosis of malaria. Since only the core of the beads is fluorescent, photobleaching and stability issues, which are often found in many fluorescent immunoassays are mitigated. The use of fluorescent beads does not present a limitation for POC applications as many handheld peripheral fluorescence readers have been developed for use at the POC. The attenuation of fluorescence intensity emitted from the core of the beads due to formation of a silver film alleviates the need for fully transparent devices. The assay achieved a sensitivity below 6 ng mL\(^{-1}\) of PfHRP2, which is in line with the desired sensitivity specified in the TPP for malaria elimination settings. The fluctuation in temperature should not cause any adverse effects on silver staining. The short turnover time of 20 min supports the use of this technology in the field where health workers often have to perform many tests.
in a day, or when patients have to receive treatment within the same visit. The microfluidic prototype reported here relies on capillary forces to facilitate sequential chemical analysis without any active elements. This makes the microfluidic chips compact and easy to use while retaining all advantages of rapid tests.

There are three tasks that need to be performed to transform the promising prototype developed in this work into a commercial product. First, integration of all reagents into the capillary-driven platform will greatly simplify the test handling for end users. This can be achieved by chemically converting reagents into dissolvable dry forms that can be stored on chip. The second step is to fabricate the chips in plastic, for example, using injection molding or hot embossing. Recent advancements in these technologies ensure that even the smallest microfluidic features of up to 5 µm can be produced with high resolution. Last but not least, a low-cost fluorescent detection reader can be developed using smartphones. In our point of view, these remaining problems are at the engineering level. We believe that the combination of capillary-driven microfluidics, integration of reagents needed for the IGSS into the same platform, and interfacing the plastic chip with smartphone-based readout can greatly decentralize the fight against malaria and turning the ambitious goal of eliminating this scourge into a historical milestone for the global public health.
5.2 Outlook

Different contexts require different TPPs; therefore no single diagnostic tool could help accelerate the elimination of malaria. State-of-the-art research suggests a potential for combining microfluidics-based diagnostics with smartphone-based readouts as an ideal platform for point-of-care diagnostics. This combination has led to promising prototypes. For the case management scenario, a handheld diagnostic device which includes a microfluidic-based immunoassay and a phone-based readout could provide not only more accurate analysis than current RDTs, but would also be better harmonized with the national malaria programs. For the surveillance scenario, where the last parasite needs to be traced, microfluidic-based molecular detection platform interfaced with smartphone-based readouts could be ideal. To facilitate the realization of this ideal field-suitable diagnostic tool, it is crucial to call for close collaborations between technology developers and malaria experts, partnership between private and public sectors, appropriate funding mechanism and sustainable political support.

Mobile health applications have rapidly been growing in recent years and there is a trend in interfacing consumer electronics such as smartphones with lateral flow RDTs or microfluidic-based devices [186], [187]. Such combination is expected to deliver increased objectivity of test result interpretation and improved connectivity of the entire healthcare systems. The automation and digitized test results can be more easily combined with other
health related parameters and combined with medical decision support systems. User-friendly interfaces, automated result analyses, remote-monitoring and data aggregation, and active quality assurance are just a few additional benefits of this approach [188].

In 2008, paper-based microfluidics were integrated with a smartphone camera to perform immunoassays [189]. The camera of the phone was used to take a photograph of the detection zone before and after the deposition of specimen. Since then, many groups have started to develop and enhance capabilities of phone-based low cost diagnostic readers [187].

Table 9 presents an overview of recent work in developing phone-based prototypes that can be used to detect variety of biomarkers for a wide range of diseases with clinically relevant performance, for instance PfHRP2 detection [190], genomic DNA from *Escherichia coli* [191]. Devices are designed for a broad spectrum of applications, from cancer detection [192] to personalized food allergen monitoring [193]. A wide range of strategies are also derived to enhance signal strength, for instance, using quantum dots [194], Rayleigh/Mie scatter [195], or gold nanoparticles [196], and others [197], [198], [199], [200], [201]. At present, applications of smartphone-based diagnostics for malaria detection can be divided into categories: phone-based RDT readers, which provides automatic interpretation of results, and phone-based brightfield microscopes, which allow simple and portable means to visualise parasites in blood samples (Table 10) [202].
<table>
<thead>
<tr>
<th>Optical detection</th>
<th>Data analysis</th>
<th>Signal transduction</th>
<th>Target biomarker</th>
<th>Sample</th>
<th>Platform</th>
<th>Performance</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phone LED and camera + 4 external lenses and mirrors</td>
<td>Mie scattering simulation online</td>
<td>Immunoagglutination (Mie light scattering)</td>
<td>P f HRP malaria biomarker</td>
<td>Human blood</td>
<td>Microbeads</td>
<td>1 pg/mL - 10 ng/mL</td>
<td>[190]</td>
</tr>
<tr>
<td>Computational power + external optical fiber + LED</td>
<td>Phone application</td>
<td>Fluorescence</td>
<td>Genomic DNA</td>
<td>Escherichia coli and Staphylococcus aureus</td>
<td>Microfluidics</td>
<td>Comparable to that of commercial PCR</td>
<td>[191]</td>
</tr>
<tr>
<td>Phone camera</td>
<td>Phone app</td>
<td>Colorimetry</td>
<td>HE4 (ovarian cancer biomarker)</td>
<td>Urine</td>
<td>Microchip</td>
<td>89.5% sensitivity, 90% specificity</td>
<td>[192]</td>
</tr>
<tr>
<td>2 external LEDs + phone camera</td>
<td>Phone app</td>
<td>Colorimetry</td>
<td>Peanut</td>
<td>Cookies</td>
<td>Sample holder</td>
<td>&lt; 1ppm</td>
<td>[193]</td>
</tr>
<tr>
<td>External LED + phone camera + additional lens</td>
<td>Phone application</td>
<td>Fluorescence</td>
<td>Escherichia coli</td>
<td>Milk, water</td>
<td>Glass capillary</td>
<td>5 - 10 cfu/mL</td>
<td>[194]</td>
</tr>
<tr>
<td>External LED and optical fibers</td>
<td>Phone app</td>
<td>Immunochromatography (Mie scatter)</td>
<td>Thyroid stimulating hormone</td>
<td>Human serum</td>
<td>Nitrocellulose test strip</td>
<td>0.31 mIU/L</td>
<td>[195]</td>
</tr>
<tr>
<td>Phone camera + external LED</td>
<td>Computer</td>
<td>Colorimetry</td>
<td>Human IgG</td>
<td>Human IgG sample</td>
<td>Microfluidics, silver deposition</td>
<td>n/a</td>
<td>[196]</td>
</tr>
<tr>
<td>Snap-on attachment (lens + LEDs) + phone camera</td>
<td>Phone app</td>
<td>Immunochromatography</td>
<td>Malaria biomarkers</td>
<td>Whole blood</td>
<td>Rapid test diagnostic strips</td>
<td>4 x dilution c.f. RDTs</td>
<td>[197]</td>
</tr>
<tr>
<td>3 external attachments + lenses + LED + phone camera</td>
<td>Phone application</td>
<td>Fluorescence</td>
<td>Cell count</td>
<td>Blood</td>
<td>Sample holder</td>
<td>600 - 2500 white cells/image</td>
<td>[198]</td>
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<td>Phone camera</td>
<td>Phone app</td>
<td>Colorimetry</td>
<td>pH</td>
<td>Test strip</td>
<td>n/a</td>
<td>400 - 700 red cells/image</td>
<td>[199]</td>
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<td>External LEDs and photodiode</td>
<td>Phone app</td>
<td>Colorimetry</td>
<td>Glucose</td>
<td>Urine</td>
<td>Paper strips</td>
<td>0 - 250 mg/dL</td>
<td>[200]</td>
</tr>
<tr>
<td>Snap-on attachments (lens + LED) + phone camera</td>
<td>ImageJ on computer</td>
<td>Fluorescence</td>
<td>Prostate specific antigen (PSA)</td>
<td>Whole blood</td>
<td>Microfluidics</td>
<td>Dynamic range 0.08 - 60 ng/mL</td>
<td>[201]</td>
</tr>
</tbody>
</table>

Table 9: Examples of lab-on-a-phone applications
A smartphone was used for quantitative reading of the Optimal-IT test, a commercially available malaria RDT with a snap-on unit as reader that is suitable for both Android and iPhone [197]. Images of RDTs were acquired, in either transmission or reflection, and then processed in real time to deliver test results within 10 minutes. The spatio-temporal information collected by this device can document prevalence of many infectious diseases and would allow efficient tracking of epidemics. Another approach to integrate a custom microfluidic-based immunoassay detecting PfHPR2 with phone-based detection was the development of a microfluidic chip, which can be connected to a phone camera to analyze signals and deliver results in 10 minutes. The opto-mechanical unit in this case consisted of optical fibers, microfluidic chips and mirrors, and could be easily removed from the back camera of the phone. The principle was to quantify changes in fluorescent intensity upon capturing of PfHPR2 on the sensing region, yielding a LOD of 1 pg/mL of PfHRP2.

<table>
<thead>
<tr>
<th>Approach</th>
<th>Working principle</th>
<th>Advantage(s)</th>
<th>Disadvantage(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lens-free</td>
<td>Using post-processing techniques of images collected by phone camera.</td>
<td>Comparable FOV - independent resolution to traditional brightfield microscopy.</td>
<td>Requiring sufficient processing power. Close placement of sample to camera.</td>
</tr>
<tr>
<td>On-lens</td>
<td>Using reflective element attached to phone camera at focus, or ball lens mounted in front of camera lens.</td>
<td>Low cost and comparable resolution to other phone-based microscopies.</td>
<td>Only allows for a small FOV of captured images to be in focus. Out of focus images needed to be adjusted.</td>
</tr>
<tr>
<td>Opto-mechanical</td>
<td>Using additional snap-on units that house optical components</td>
<td>Portable, low cost and easy to fabricate.</td>
<td>Dependence of attachment's design on phone's configuration.</td>
</tr>
<tr>
<td>attachment</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Table 10: Key characteristics of common imaging technologies for phone-based microscopy
adapted from [202]
in 10% diluted blood [190]. Several examples such as lateral-flow based RDT reader [197], embedded electrical detection [137] or smart dongle [84] are summarized in Figure 31.

1) Lateral-flow based RDT reader

2) Microfluidics-based RDT readers

Figure 31: Examples of integrated rapid diagnostic test reader on a cell phone

(1) Lateral-flow based RDT reader [197] and (2) Microfluidics-based RDT readers (2a) [137], (2b) [84].

Accurate and consistent blood smear reading is challenging to attain in health centres or small clinics in remote regions. A phone-based microscope is a low cost option that can offers enhanced image quality, improved accuracy and user comfort [198], [203]. There are two simplified imaging techniques suitable for smartphone apps: 1) lens-free holographic imaging [204], and 2) on-lens devices [205], [190] (Figure 32).
Holography is an image-constructing technique using scattering and interference of light and pixel super-resolution to enhance optical images [204]. An automated lens-less holography was developed with a sufficient field of view of 24 mm$^2$ to visualize and capture images of P. falciparum in blood smears [202].

Phone-based microscopy can also be engineered to be a field-ready polarized light microscope without compromised fidelity and resolution [206]. The principle was to detect light birefringence caused by the crystallization of hemozoin. This field-based, modular microscope could magnify P. chabaudi parasites up to 50 times, gaining a comparable performance compared to conventional polarized microscope. Additional benefits of this prototype are simple operations and low cost per test. Further work using clinical samples...
could confirm the full potential of this novel phone-based polarized light microscope.

In summary, there are a lot of promising new developments in the diagnostic area. Our approach in this thesis was to develop a self-driven microfluidic rapid diagnostic test for malaria integrated on a silicon chip where the use of an ISGG assay provides high sensitivity. To bring this prototype to market, we envisage the use of smartphones as test readers and the replacement of the silicon chip by cheaper plastic ones. This approach and other similar projects listed above could potentially represent the next generation of rapid diagnostic tests. It is hoped that the ongoing research on POC diagnostics in low resource settings can help solve major public health issues like malaria in the near future.
List of References


2, pp. 82–99, 2008.


[107] E. Banoth, V. K. Kasula, V. K. Jagannadh, and S. S. Gorthi,


[113] S. Yang, A. Undar, and J. D. Zahn, “A microfluidic device for


[137] P. B. Lillehoj, M.-C. Huang, N. Truong, and C.-M. Ho, “Rapid


[205] I. Navruz, A. F. Coskun, J. Wong, S. Mohammad, D. Tseng, R. Nagi,

List of Publications

Related to this thesis

Refereed Journal Publications


Conference oral presentations


Not related to this thesis

Conference oral presentations

1. G. Pham, M.M. Gong, B.D. MacDonald, T.V. Nguyen, D. Sinton, Development of a biochemistry assay to assess liver health on a microfluidic device, 7th Annual Ontario-on-a-Chip, May 2012, and MIE Research Symposium, June 2012. [G. Pham awarded "Innovation most likely to be commercialized" poster award at Ontario-on-a-chip]
Conference poster presentations

2. B.D. MacDonald, M.M. Gong, G. Pham, T.V. Nguyen, D. Sinton, Side-by-side testing of a high throughput blood separation device at the National Hospital for Tropical Diseases, 7th Annual Ontario-on-a-Chip, May 2012, and MIE Research Symposium, June 2012. (Poster presentation)


5. M.M. Gong, B.D. MacDonald, G. Pham, T.V. Nguyen, D. Sinton, Sample collection and processing for multiplexed blood-based point-of-care analysis, Grand Challenges Meeting, Grand Challenges Canada, New Delhi, India, November 2011. (Poster presentation by M.M. Gong)
# Gemma (Minh Ngoc) PHAM

## EDUCATION

<table>
<thead>
<tr>
<th>Date</th>
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<th>Institution</th>
<th>Country</th>
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<td>03.2014 - present</td>
<td>PhD. Department of Health Sciences and Technology</td>
<td>Swiss Federal Institute of Technology (ETH) Zürich</td>
<td>Switzerland</td>
</tr>
<tr>
<td>Thesis: <strong>A rapid point-of-care test for malaria diagnosis in elimination settings in Vietnam</strong></td>
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<td>• Developed a highly sensitive, rapid microfluidics-based diagnostic prototype for malaria detection in low resource settings</td>
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<tr>
<td>2010 - 2012</td>
<td>MASc. Mechanical &amp; Industrial Engineering Dept.</td>
<td>University of Toronto</td>
<td>Canada</td>
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<tr>
<td>• Developed prototypes for a blood filtration device for low resource settings and attained CAD$1m worth of funding from Grand Challenges Canada.</td>
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<td>• Assisted in securing CAD$500’000 funding for a study of infertility using microfluidics-based IVDs.</td>
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<tr>
<td>2004 - 2009</td>
<td>BSc. &amp; MSc. Department of Chemistry</td>
<td>Imperial College London</td>
<td>United Kingdom</td>
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<tr>
<td>• Including one year training in industry at Brand Technical Centre, Diageo Scotland.</td>
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## EXPERIENCE

<table>
<thead>
<tr>
<th>Date</th>
<th>Position</th>
<th>Institution</th>
<th>Location</th>
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<tr>
<td>• Coordinate evaluations required for the WHO prequalification (PQ) of in vitro diagnostics (IVDs) assessments.</td>
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<tr>
<td>• Establish productive working relationships with manufacturers, international procurement agencies and national governments regarding PQ of IVD products.</td>
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<tr>
<td>03.2014 - present</td>
<td>Research Assistant</td>
<td>ETH Zürich &amp; IBM Research</td>
<td>Zürich, Switzerland</td>
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<tr>
<td>• Analysed the landscape of in vitro diagnostics technologies how advanced technologies can help with the development of point-of-care diagnostics.</td>
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<tr>
<td>• Established collaborations with Swiss Tropical and Public Health Institute (Swiss TPH), the National Institute for Malariaology, Entomology and Parasitology of Vietnam, IBM Research Zürich and ETH Zürich on developing and validating microfluidics-based</td>
<td></td>
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</table>
IVDs for malaria detection.

10.2016 – 03.2017  **Graduate intern**  
WHO Essential Medicines and Health Products  
Geneva, Switzerland  
- Conducted landscape analysis for point-of-care diagnostics available for Antimicrobial Resistance (AMR) to provide an overview of IVDs available for AMR to high level meetings with international organizations and agencies.  
- Finalized the 2016 compendium for innovative health technologies for low-resource settings.

2014  **Business proposal evaluator**  
Innovation Prize for Africa  
Switzerland  
- Evaluated 90 market-oriented business solutions which competed for the funding award of USD $100'000 to promote sustainable developments in Africa.

2013 - 2014  **Executive Assistant**  
Vietnam Medical Equipment and Chemicals JSC  
Vietnam  
- Conducted market research to evaluate how medical devices and equipment are procured in public and private sector in Vietnam.

**PUBLICATIONS**


**AWARDS AND SCHOLARSHIPS**

2015  Swiss South African Joint Research Program, Switzerland

2014 - 2017  Engineering for Development Doctoral Scholarship, Sawiris Foundation, ETH Global (2 awards per year globally)

2012  “Innovation most likely to be commercialized” Poster Award, Hill & Schumacher Patent & Trademark Agents, 7th Annual Ontario-on-a-chip
<table>
<thead>
<tr>
<th>Year</th>
<th>Award Description</th>
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<tbody>
<tr>
<td>2008</td>
<td>Masters Research Award, Diageo Scotland</td>
</tr>
<tr>
<td>2004 - 2007</td>
<td>Undergraduate Scholarship, Vietnam Ministry of Education and Training</td>
</tr>
</tbody>
</table>

**LEADERSHIP**

**2017**  
**Academic Jury**  
St Gallen Wings of Excellence Award  
- Evaluated final top 6 essays themed “The dilemma of disruption” to promote social and liberal economic order.

**Delegate**  
International Student Forum: Healthcare & Global Economy  
- Discussed pressing needs for healthcare services in developing countries, including political and technical commitments to raise awareness at the global level.

**LANGUAGES & SKILLS**

- English and Vietnamese (advance/mother tongue)  
- German (A1.2/2.1)  
- Project management

**INTERESTS**

- Sky-diving, scuba-diving, paintings, global health and international politics