Empowering the Experimental Biology of Plasmodium Vivax Through Elucidating Requirements for Ex Vivo Culture

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Empowering the experimental biology of \textit{Plasmodium vivax} through elucidating requirements for \textit{ex vivo} culture

A dissertation presented

by

Gabriel W. Rangel

to

The Committee on Higher Degrees in Biological Sciences in Public Health

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in the subject of

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Empowering the experimental biology of *Plasmodium vivax* through elucidating requirements for *ex vivo* culture

**ABSTRACT**

For over 100 years, various research groups have been attempting to establish a continuous and robust *in vitro* culture system for the blood stages of various *Plasmodium* species, apicomplexan parasites that cause malaria, which is a testament to the potential importance of *in vitro* culture. Indeed, for *P. falciparum*, *in vitro* culture of blood stages has enabled countless experimental capabilities including large-scale antimalarial screens, vaccine development and production, and malaria transmission studies. However, there still exists no comparable *in vitro* culture system for *P. vivax*, greatly setting back the fight against this malaria parasite that puts one third of the global human population at risk for infection. Thus, this dissertation aimed to better understand and overcome various aspects of *P. vivax* intraerythrocytic biology that act as hurdles on the path toward culture.

In Chapter One, we review the current state of knowledge surrounding *P. vivax*, its impact on global health and malaria eradication efforts, the path toward *in vitro* culture of *Plasmodium* parasites and why this is a particularly difficult but necessary goal for *P. vivax*. In Chapter Two, attempting to maximize the experimental utility of cryopreserved *P. vivax* isolates, we initially focused on increasing the quantifiability of our assays by enriching parasites and increasing their survival through one round of maturation. We successfully applied reticulocyte enrichment techniques that minimize cellular dehydration to robustly enrich *P. vivax*-infected cells. We also identified Iscove’s Modified
Dulbecco’s Medium from a culture media screen as a formulation that doubled *P. vivax* survival to mature gametocytes and schizonts. These advancements enabled the adaptation of an isotopic metabolic labelling assay for rapid and sensitive antimalarial assays. In Chapter Three, the enhanced enrichment and survival of *P. vivax* also enabled the application of low-input RNAseq technologies to generate robust transcriptome data from minimal parasite material. From this we establish the first transcriptome from purified *P. vivax* gametocytes, identifying novel markers and potential vaccine candidates. We also conclude that culture media has a relatively small effect on parasite transcriptome signature, suggesting the key effect of the culture media may be on host cells rather than the parasite itself. In Chapter Four, we focus on the host cells and utilize a surface proteomics approach to identify novel markers of very young members of the heterogeneous circulating reticulocyte population. *P. vivax* parasites within host cells positive for one of these markers, SLC12A6, are more likely to survive intraerythrocytic maturation, and this appears to be dependent on the potassium chloride cotransporter function of this protein. Finally, Chapter Five highlights the implications of the findings of this dissertation and suggests future directions the field of *P. vivax* molecular biology should take. Overall, this work has enhanced the capacity for molecular experimentation on *P. vivax*, revealed some important interactions between parasite and host-cell biology, and moved the field closer to a continuous and robust *in vitro* culture system.
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CHAPTER ONE
INTRODUCTION

1.1 Further emphasis on *Plasmodium vivax* research is needed

1.1.1 *P. vivax* remains a significant threat to global health and development

Although much headway has been made over the past decade against the incidence and mortality caused by all human malaria parasites, the 2018 World Malaria Report from the World Health Organization suggests that progress on this front has recently stalled, with no evidence of significant reduction in global cases between 2015 and 2018 [1]. Of the human-infecting malaria parasites, *Plasmodium vivax* is the most geographically widespread, putting approximately one-third of the entire global population at risk of infection, resulting in an estimated 7.51 million cases in 2017 [1] (Figure 1.1). However, because there is no clinical diagnostic for the dormant liver forms of *P. vivax*, and because extremely low blood-stage parasitemias often make infection difficult to diagnose via microscopy, this estimation are likely a gross underestimate of true *P. vivax* infections [2, 3].
Figure 1.1 | A map of the estimated incidence of clinical Plasmodium vivax in 2017. This map is modified from the Malaria Atlas Project.
Patients infected with *P. vivax* often experience profoundly debilitating febrile episodes, respiratory difficulty, severe anemia, and pregnancy complications [4]. Because these symptoms tend to be less severe and less deadly than those caused by the more prevalent human malaria parasite, *Plasmodium falciparum*, *P. vivax* has historically been inaccurately considered “benign.” This partially explains why relatively fewer resources have been dedicated to biological research of and public health interventions for *P. vivax*. Indeed, a cursory PubMed search of either “*Plasmodium vivax*” or “*Plasmodium falciparum,*** yields a cumulative publication count of 8207 and 37757 respectively, and examining publication numbers over time clearly demonstrates the historical discrepancy between these two species (Figure 1.2). However, there is growing appreciation for the fact that *P. vivax* infections can, in fact, be quite severe, resulting in incapacitation and even culminate in death [5, 6]. In addition to the direct negative health effects on infected individuals, *P. vivax* infection has profound impacts on micro and macroeconomics as infected patients are often absent from work or school for 4-15 days, repeated infections in children has significant effects on scholastic performance, and public health systems often ultimately bear the burden of healthcare costs [7]. Altogether, it is very clear that *P. vivax* malaria remains a disease of significant global health importance.
Figure 1.2 | PubMed publications of *P. vivax* and *P. falciparum* over history. Based on the PubMed “Results by Year” option when searching “Plasmodium falciparum” or “Plasmodium vivax” in March 2019.
1.1.2 *P. vivax* and malaria eradication objectives

To date, there have been two major, global efforts to not just control malaria infections, but to eradicate all human-infecting species of the parasites from the planet. The first effort began in 1955 when the World Health Organization (WHO) decided that they:

“…should take the initiative, provide technical advice, and encourage research and co-ordination of resources in the implementation of a programme having as its ultimate objective the world-wide eradication of malaria [8].”

This first effort was successful in eliminating the disease from many geographic regions including the United States of America and much of Europe, and it was integral in informing future global efforts by the United Nations and the WHO, including the successful eradication of smallpox [9]. However, by 1969 the WHO had to recognize that due to many issues including reduction of funding, lack of public health infrastructures and suboptimal partnerships with local communities, there were regions where eradication would not be quickly feasible and shifted its strategy in those areas back to controlling infections and clinical outcomes, and this remained the primary focus for the next three decades [9].

Then, in 2007, Melinda and Bill Gates called for a refocus of antimalarial efforts onto the final goal of global eradication, saying:

“The goal of eradicating malaria has the power to create great expectations, grand efforts, and record funding. When you ask people to donate time and money to save lives, they can be very generous. When you ask them to give time and money to eradicate a disease, their generosity can multiply. Those are the benefits. They are also the risks. If high energy and high expectations don't lead to success—it saps money and morale. People give up. Governments, foundations, and corporations cut their funding, malaria surges back—and gains can be quickly wiped out. [10]"
Indeed, as evidenced by the stall in the reduction of global malaria cases during recent years, the risk of failure in eradication efforts is quite real. Furthermore, it is to be expected that *P. vivax* will be a particularly challenging obstacle in global malaria eradication.

The life cycle of *Plasmodium spp.* parasites is a very complex set of biological processes in various environments such as the human liver, human blood and *Anopheline* vector (Figure 1.3). However, compared to *P. falciparum*, *P. vivax* has many unique biological characteristics that make it more difficult to tackle from a public health perspective. First, *P. vivax* has the presence of spontaneously reactivating dormant liver stages called hypnozoites, that can remain quiescent in infected patients for up to years, producing no symptoms until they are reactivated by some currently unknown mechanism [11]. Moreover, there is no reliable clinical detection method to diagnose the presence of hypnozoites, and the only developed hypnozoitocidal drug class, 8-aminoquinolones, can produce potentially lethal hemolytic crises in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency, a genetic mutation that is present in up to 32.5% of individuals in some endemic regions, greatly complicating the administration of effective chemotherapeutics [12, 13]. Secondly, the concentration of parasites circulating in the blood is relatively low for *P. vivax* cases, resulting in many asymptomatic carriers that are undetectable by microscopy, making accurate diagnoses of carriers difficult [14]. Lastly, the transmissible forms of *P. vivax* parasites, gametocytes, develop extremely quickly, often before any potential symptoms develop, and the relatively large *P. vivax*-infected populations where no symptoms ever develop have been also shown to be infectious [14-16].
Figure 1.3 | The life cycle of *Plasmodium spp.* Microscopy images represent the various morphological stages of *Plasmodium vivax* during intraerythrocytic development. Black bars on microscopy images represent 5 µM.
Altogether, the biological aspects of *P. vivax* indicate that it will be an extreme challenge to eradicate it, and if the global effort does not increase focus on understanding the biology of this parasite, it very well may be doomed to fail again. However, increasing the biological understanding of this malaria species is exceedingly difficult due to a lack of robust *in vitro* culture systems for the blood stages of *P. vivax*.

1.2 *Plasmodium vivax in vitro* culture: A difficult goal

1.2.1 A brief history of human-infecting *Plasmodium* *spp.* and *in vitro* culture

The eukaryotic, apicomplexan parasites that are the causative agent of malaria disease were first identified by Charles Louis Alphonse Laveran in 1880, nearly 130 years ago, when he perceptively discovered the exflagellating male gametocyte forms using a relatively underpowered microscope to view the unstained blood from a post-mortem sample of a young French soldier that had died from malaria complications [17, 18]. Attempts to culture human malaria parasites began soon thereafter, but only a few cycles of *in vitro* replication had been achieved before the cultures ultimately died [19, 20]. These short-term cultures enabled various studies including the examination of antimalarial compounds, biochemical processes and immunology [21-23]. In 1976, about a century after the discovery of the causal parasite, a robust culture system was developed for the blood stages of *P. falciparum* [24]. This was accomplished by testing multiple iterations of various culture parameters, finally culminating on providing the parasites human erythrocytes in RPMI 1640 media supplemented with HEPES buffer, sodium bicarbonate and human serum cultured at 7% carbon dioxide and 1-5% oxygen gas concentrations [24]. Indeed, this culture system and variations therein have enabled numerous integral
experimental techniques over following decades, including large-scale drug screens, genetic manipulation of the parasites, vaccine development, and other molecular and cellular biology approaches [25]. More recently, adapting these protocols has also unlocked the ability to culture other malaria parasites, including strains of human-infecting *P. knowlesi* [26, 27].

Similarly, since 1912 there have been many previous efforts to establish robust culture for *P. vivax* intraerythrocytic parasites [28]. In fact, in 1997 there was a report of successful, robust culture by Golenda et al., where *P. vivax* intraerythrocytic parasitemia doubled each 48-hour replication cycle over the course of three weeks [29]. Unfortunately, this system was ultimately discontinued by the authors due to the laborious process, and, despite multiple efforts by various groups independently and in collaboration, these results have not been reproduced. The most successful recent attempts at *P. vivax* culture have centered around similar culture conditions as used by Golenda et al., including the use McCoy's 5A media but with various combinations of media supplements [30, 31].

### 1.2.2 Establishing *P. vivax* in vitro culture would greatly accelerate eradication efforts.

If the malaria research community had available a continuous and robust *in vitro* culture system for *P. vivax*, the advancement of research toward novel treatments, vaccines and general eradication goals would be enormously accelerated in many ways. First, an *in vitro* culture system that allows for the continual expansion of *P. vivax* intraerythrocytic parasites would provide enough biomass to consider experimental
approaches that are currently unattainable with present culture systems. For instance, it would be possible to perform large-scale whole-parasite drug screens, an approach routinely applied to *P. falciparum* using various readout techniques such as isotopic and/or fluorescent labeling of parasite nucleic material or quantification of parasite metabolic biproducts like beta-hematin [32-35]. Large-scale screening has been remarkably successful with the *P. falciparum* parasite, where screens of millions of small molecules can be achieved, leading to the discovery of potentially useful antimalarial compounds, many of which are in current preclinical or clinical trials [36-38]. Another example of experimental tool that requires a robust culture system is parasite genetic manipulation. Being able to create genetic mutants of *P. vivax* would facilitate, for instance, identifying drug targets or creating reporter strains, which have been integral tools to *P. falciparum* researchers [39].

Another field of research that would benefit from robust *in vitro* culture of *P. vivax* intraerythrocytic stages would be vaccine development. Directly, being able to cultivate blood-stage parasites would enable development and production of whole-killed blood-stage vaccines, which have already been shown to protect against blood-stage challenge and inhibit transmission using mouse models [40, 41]. Indirectly, being able to rear transmissible blood-stage *P. vivax* parasites would greatly facilitate controlled infections of mosquitoes, which is an important experimental outcome in itself, but which also is necessary to develop sporozoite-based vaccines, an approach that is currently under development for *P. falciparum* with promising results [42].

Finally, having an *in vitro* culture system for intraerythrocytic *P. vivax* would unlock the ability of many more research groups to contribute toward the understanding *P. vivax*
biology. Currently, it is possible to design experiments using available short-term culture techniques; however, as discussed above, the options for experimentation are greatly limited given the system. Furthermore, experimentation is extremely resource-intensive, necessitating research groups to establish relationships and/or infrastructure to obtain primary isolates from either patients or animal models. If there were culture-adapted lines of *P. vivax*, this barrier would be lifted, and many more research groups could apply their expertise to investigate parasite biology. Still, *P. vivax in vitro* culture would likely still be relatively resource-intensive due to the reticulocyte tropism of *P. vivax*, discussed later, which requires the sourcing and enriching of suitable host cells. However, *in vitro* adaptation of the reticulocyte-tropic human malaria parasite, *P. knowlesi*, to invade mature erythrocytes has been reported [27]. This may be possible with *P. vivax* once long-term culture in reticulocytes is established and would further reduce the cost of *P. vivax* experimentation, unlocking this organism for research by even more groups.

1.2.3 Unique *P. vivax* intraerythrocytic biology complicates *in vitro* culture

There are many reasons that a continuous and robust *in vitro* culture system for *P. vivax* has remained elusive. Inherently, in order to establish *in vitro* culture, researchers must begin with primary isolates from infected individuals or animal models. Obtaining clinical isolates is resource-intensive and requires a research group to have access to not only a clinical infrastructure and a patient population that is willing to coordinate and collaborate with research efforts but also access to a nearby research laboratory facility that is capable of enabling sample processing for cryopreservation at minimum and ideally capable of supporting full-scale experimentation. Finding or establishing
satisfactory clinical and laboratory environments is particularly difficult and costly due to the fact that many of the regions endemic for \textit{P. vivax} are also resource-poor settings [43]. However, the logistics of obtaining samples is not the only obstacle to establishing \textit{P. vivax} in vitro culture. \textit{P. vivax} is unique from \textit{P. falciparum} in many aspects of its intraerythrocytic biology, and many these aspects further complicate the establishment of short-term and long-term in vitro culture systems [44].

First, circulating parasitemias from \textit{P. vivax} infections tend to be relatively low, greatly complicating the quantification of any short-term assays or the monitoring of in vitro survival and/or growth of long-term assays. \textit{P. falciparum}-infected patients often have circulating parasitemias over 0.5\% on average, and “high level of infection” is sometimes defined as above 1\% [45, 46]. Meanwhile, \textit{P. vivax}-infected patients often have parasitemias below 0.1\% and are rarely above 0.5\%, which is challenging to accurately quantify by the standard light microscopy methods [4, 46, 47]. While some \textit{P. vivax} enrichment protocols have been established to enrich parasites from clinical isolates, namely density-based assays using Percoll diluted with phosphate buffered saline (PBS), they often are inconsistent in their performance and also require relatively large amounts of precious starting material [48]. This unreliability in enrichment protocols results in the loss of utility of many isolates, even for short-term assays, further increasing the cost to achieve effective \textit{P. vivax} in vitro results.

Second, \textit{P. vivax} apparently has a significantly different culture media requirement compared to \textit{P. falciparum}. Even when obtaining ex vivo \textit{P. vivax} parasites that invaded their preferred host cells within the natural host, \textit{P. vivax} survival in RPMI is relatively poor, and the current standard \textit{P. vivax} culture medium, McCoy’s 5A, also results in either
extremely unstable parasite growth and/or the dramatic loss of parasite concentration over the course of intraerythrocytic maturation cycles [49-51]. While there is much known about the requirements of in vitro P. falciparum when it comes to the specific nutrients, osmolality, ion concentration and acidity of the culture medium, little remains understood about those parameters for in vitro P. vivax [52-54]. Nevertheless, optimizing ex vivo survival of P. vivax over the course of even one intraerythrocytic development cycle (IDC) is essential to maximizing the utility of costly clinical or animal model isolates for antimalarial, immunological and other molecular cellular assays, and is important even if robust in vitro culture is never attained.

A third biological challenge of P. vivax is the strong preference for invading the youngest circulating host red blood cells called reticulocytes, which generally account for less than 2% of circulating erythrocytes within a healthy blood donor [55]. This restriction for reticulocytes is even more complicated when the heterogeneity of the reticulocyte population is considered [56]. Circulating reticulocytes are defined by the presence of a reticulum, which largely consists of residual ribosomes and associated RNA [57]. Indeed, this cell population ranges from extremely young enucleated erythroblasts that have just egressed from the bone marrow to cells that have are finishing the degradation and autophagy of their reticulum, a maturation process that happens over the course of approximately 12-48 [58]. During this maturation, many changes to the reticulocyte occur, including major changes in surface proteins, membrane composition, morphology, cytoskeletal organization and the degradation of remaining organelles [59-62]. Indeed, it has been shown that of this heterogeneous reticulocyte population P. vivax preferentially invades the youngest subset as defined by the relatively high surface expression of
transferrin receptor (TfR), also known as CD71 [63-66]. However, it is also clear that even when giving CD71-high cells for *P. vivax* invasion, invasion efficiency is relatively low as is subsequent survival through maturation, which suggests that *P. vivax* may require an even more specific subset of reticulocytes for robust propagation.

Finally, the reticulocyte restriction of *P. vivax* means that purifying or enriching invadable host reticulocytes is absolutely necessary for maintaining *P. vivax* survival in *in vitro* culture systems. Furthermore, the rarity of these invadable reticulocytes in healthy donors means that significantly more donor material is required to obtain necessary numbers of host cells for *P. vivax in vitro* culture. Some groups have reported relative success using blood sources that are inherently enriched in reticulocytes. These sources include blood from patients with hemochromatosis, blood from umbilical cords of newborn donors, and reticulocytes from hematopoietic stem cells that were differentiated *in vitro* [29, 51, 65, 67, 68].

Once the source of reticulocytes is obtained, there are a few established methods of enriching or purifying reticulocytes and reticulocyte subpopulations. Perhaps the most commonly used methods are variations of density-based enrichment using Percoll or dextran/Ficoll gradients [69-71]. Another approach is to capitalize on the surface expression of CD71 on the young reticulocyte population and mark reticulocytes with anti-CD71 magnetic beads and enrich this cell population using magnetic-activated cell sorting (MACS) [64, 72]. Similarly, fluorescence-activated cell sorting has also been used to isolate reticulocytes by staining for either the reticulum itself, CD71 or both [73]. Applying FACS techniques is particularly useful as reticulocytes of various developmental states
can be specifically sorted based on various markers including the reticulum, CD71, CD49d and Band3 [56, 64, 74].

In all, just as with the early, short-term culture systems for *P. falciparum*, short-term cultures of *P. vivax* have unlocked the ability apply some experimental techniques and investigate some aspects of parasite biology; however, the availability of applicable techniques is limited by low parasite biomass and parasite intractability [73, 75, 76]. Furthermore, the range of research groups that are capable of performing the existing techniques is extremely limited by the high cost of *P. vivax* sample acquisition, processing and culture conditions. Each of these limitations would be quickly lifted if an *in vitro* culture system is established for intraerythrocytic *P. vivax*, making the establishment of culture an important goal. However, it is also clear, given the century of unsuccessful pursuit, that this is an ambitious goal that would benefit from segmentation into smaller, attainable aims.

1.3 Summary of aims

The goal of this dissertation is to understand better the metabolic requirements and host cell niche of intraerythrocytic *P. vivax* in order to empower experimental biology and move toward continuous and robust *in vitro* culture. In Chapter Two, we address two major biological challenges of using *P. vivax* clinical samples, extremely low circulating parasitemias and poor *ex vivo* survival within the first round of maturation. By capitalizing on the reticulocyte tropism of *P. vivax* and previously established reticulocyte enrichment protocols, we report a robust method of enriching small-volume clinical isolates using a Percoll density gradient buffered with a solution high in potassium chloride (KCl) to
prevent cellular dehydration. We then perform a *P. vivax* maturation screen of various culture media with a focus on media designed for hematopoietic cells and found that Iscove’s Modified Dulbecco’s Medium (IMDM) performed the best. We further optimized other culture parameters, such as oxygen tension and hematocrit, and utilized the improved working parasitemias and survival to adapt an isotopic metabolic labeling-based maturation assay for rapid and sensitive antimalarial assessment.

In **Chapter Three**, we adapt an RNAseq approach designed for single-cell mammalian transcriptomics to establish robust transcriptome data of three separate *P. vivax* clinical isolates over the course of the IDC in four different culture media. From these data, we confirm that the vast majority of transcriptome variability over the IDC is accounted for by developmental stage, and that while each follows the same overall transcriptomic trajectory over the IDC, the separate patient isolates begin at different places along the early IDC transcriptomic course. Furthermore, there is little *P. vivax* transcriptomic variation accounted for by using different culture media. Next, we apply FACS to purify various stages with different nucleic acid content within a time point to the establish the first transcriptome from purified *P. vivax* gametocytes. Finally, we demonstrate variation between schizonts from different isolates in their expression of various known and putative invasion ligands.

**Chapter Four** explores the host cellular niche of intraerythrocytic *P. vivax* among the heterogeneous reticulocyte population. The first goal was to identify novel reticulocyte surface markers through the FACS-purification of circulating reticulocytes of various developmental stages and subsequent comparative surface proteomics. From a candidate list of novel very young reticulocyte surface markers, we identify one,
SLC12A6/KCC3, as more likely to be present on *P. vivax*-infected cells compared to CD71, the current standard very young reticulocyte marker. Furthermore, we show that *P. vivax* within cells positive for SLC12A6 are more likely to survive the IDC than *P. vivax* within cells negative for SLC12A6, and that this protective effect can be abrogated with a KCC-inhibitor.
1.4 References


CHAPTER TWO

Enhanced ex vivo Plasmodium vivax intraerythrocytic enrichment and maturation for rapid and sensitive parasite growth assays

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Author Contributions: G.W.R, M.A.C, U.K, and M.T.D. designed the research; G.W.R, M.A.C, and U.K performed the experiments (G.W.R.: Percoll enrichments, media screen, growth condition optimization, hypoxanthine metabolic labelling assay development, and antimalarial sensitivity studies, M.A.C.: Percoll enrichment assay development, U.K.: Percoll enrichments); C.L. and K.S.S. provided helpful discussion and insight; M.J.M. and M.U.F. organized and performed Brazilian sample collections; A.M., L.C, E.G. and P.K.R. organized and performed Indian sample collections; and G.W.R. and M.T.D. wrote the manuscript. This chapter is adapted from the publication Rangel et al. 2018, AAC.
2.1 Abstract

*Plasmodium vivax* chloroquine resistance has been documented in nearly every region endemic for this malaria-causing parasite. Unfortunately, *P. vivax* resistance surveillance and drug discovery is challenging due to low parasitemias of patient isolates, and poor parasite survival through *ex vivo* maturation, that reduce the sensitivity and scalability of current *P. vivax* antimalarial assays. Using cryopreserved patient isolates from Brazil and fresh patient isolates from India, we established a robust enrichment method for *P. vivax* parasites. We next performed a media screen for formulations that enhance *ex vivo* survival. Finally, we optimized an isotopic metabolic labelling assay for measuring *P. vivax* maturation and sensitivity to antimalarials. A KCl Percoll density gradient enrichment method increased parasitemias from small-volume *ex vivo* isolates by an average of >40-fold. Using Iscove’s Modified Dulbecco’s Medium for *P. vivax* *ex vivo* culture approximately doubled parasite survival through maturation. Coupling these with $^3$H-hypoxanthine metabolic labeling permitted sensitive and robust measurement of parasite maturation, which was used to measure the sensitivities of Brazilian *P. vivax* isolates to chloroquine and several novel antimalarials. These techniques can be applied to rapidly and robustly assess the *P. vivax* isolate sensitivities to antimalarials for resistance surveillance and drug discovery.
2.2 Introduction

*Plasmodium vivax* is the most geographically widespread malaria-causing parasite with the World Health Organization (WHO) estimating approximately one third of the global population at risk for infection, and that in 2016 approximately 8.5 million people suffered from *P. vivax* clinical infections [1]. Standard treatment for *P. vivax* infection includes chloroquine to clear asexual intraerythrocytic stages [2]; however, chloroquine-resistant *P. vivax* strains have been discovered in nearly every region with endemic *P. vivax* [3].

Clearly, novel therapeutic options are needed; however, addressing this major global health problem is made difficult by the lack of a continuous *in vitro* culture system for intraerythrocytic *P. vivax* [4]. Although, few reports have described long-term *in vitro* *P. vivax* culture in either human or animal model host erythrocytes [5-7], currently the only practical and reproducible way to test effectiveness of chemotherapeutics against *P. vivax* parasites in human host erythrocytes is to use *ex vivo* human patient isolates [8]. However, patient isolates present challenges, including often immeasurably low parasitemias, asynchronous parasite populations and significant death of parasites during *ex vivo* intraerythrocytic maturation. Furthermore, the standard assay to measure antimalarial efficacy against *P. vivax* is a modification of the laborious microscopy-based WHO microtest, requiring highly-trained microscopy technicians [9].

In this work, we systematically address these challenges. We adapted reticulocyte enrichment protocols to develop a reproducible Percoll density gradient *P. vivax* enrichment method [10]. Next, we performed a culture media screen that identified a formulation that roughly doubles parasite survival through intraerythrocytic maturation.
Finally, we established a rapid and sensitive protocol for the measurement of intraerythrocytic *P. vivax* maturation for both surveying isolates in batch for chloroquine resistance as well as for testing sensitivity to a wide range of antimalarials in development.
2.3 Materials and Methods

2.3.1 Ethical Approval

Informed consent was obtained from all patients. Study protocols for Brazilian parasite sample collection were approved by the Institutional Review Board of the Institute of Biomedical Sciences, University of São Paulo, Brazil (1169/CEPSH, 2014). For collection and use of Indian samples, the human subject protocol was approved by the ethics boards at Goa Medical College and Hospital (no number assigned), the University of Washington (42271), and the Division of Microbiology and Infectious Diseases of the National Institutes of Health (11-0074). The overall research program, under which the Indian parasites were collected, was also approved by the Government of India Health Ministry Screening Committee.

2.3.2 Parasite Sample Collection

Brazilian *P. vivax* samples were collected with protocols described elsewhere, but in the town of Mâncio Lima, Acre State [11], performed in the context of a randomized, open-label clinical trial (NCT02691910). Indian *P. vivax* samples were collected with similar protocols but using CF11 columns for leukodepletion, essentially as described previously [12]. This occurred at Goa Medial College and Hospital in Bambolim, Goa, in conjunction with the Malaria Evolution in South Asia International Center of Excellence in Malaria Research and the University of Washington.
2.3.3 Parasite Enrichment

Percoll gradients were prepared as described elsewhere [10]. Briefly, a 10X KCL stock buffer was made as 100 mM HEPES, 1150 mM KCl and 120 mM NaCl in water, and 100% KCl Percoll was made by diluting 10X KCL stock buffer 1:9 with stock Percoll (GE Healthcare Life Sciences; Pittsburgh, PA). Dilution Buffer was made as 20 mM HEPES, 1 mM MgCl$_2$, 1 mM NaH$_2$PO$_4$, 10 mM glucose, 0.5 mM EGTA, 115 mM KCl, and 12 mM NaCl in water, pH-adjusted to 7.4. 1.080 g/mL KCl Percoll was made by mixing 100% KCl Percoll with Dilution Buffer at a ratio of 64.03% to 35.97%, and density was verified at ambient temperature using a DMA 35 Portable Density Meter (Anton Paar; Graz, Austria). Cryopreserved parasites were thawed as reported previously [11]. 100 µL-1500 µL packed, parasitized blood was resuspended to 3 mL using incomplete Iscove’s Modified Dulbecco’s Medium (IMDM) and layered on 3 mL 1.080 g/mL KCl Percoll at ambient temperature in a 15 mL conical tube. This was centrifuged at ambient temperature for 15 minutes at 1200 x g with slow acceleration and no brake. The interface was removed, washed in incomplete IMDM and applied to assays.

2.3.4 P. vivax ex vivo culture

15 units of human AB+ heat-inactivated serum were acquired from Interstate Blood Bank, Inc. (Memphis, TN), pooled and stored in 50 mL aliquots at -20°C until use. Culture media was acquired commercially and prepared as described in Tables 2.1 and 2.2. Enriched parasites were washed once in respective media, plated in technical duplicate. Standard culture conditions were as follows unless otherwise noted: 96-well or ½-area 96-well flat-bottom plates, 1% hematocrit in 100 µL initial volume, 37°C in 5% CO$_2$, 1%
O₂, 94% N₂. Cytospins were made after 44 hours of maturation, methanol-fixed, then stained using Hemacolor®. Parasitemia and staging were assessed via light microscopy and whole-field counting [13].
<table>
<thead>
<tr>
<th>Culture Media Formulations for Media Screens</th>
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<tbody>
<tr>
<td>Media Supplier &amp; Product Number</td>
</tr>
<tr>
<td>10 mL Complete Media</td>
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<tr>
<td>Glasgow's Minimal Essential Media (GMEM)</td>
</tr>
<tr>
<td>L-Glutamine, Gibco 11710-035</td>
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<tr>
<td>7.79 mL GMEM, 1 mL TPB, 200 µL 1.25 M HEPES, 10 µL 50 mg/mL Gentamicin, 1 mL AB+ Serum</td>
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<tr>
<td>Basal Eagle Media (BME)</td>
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<tr>
<td>Gibco 21010-046</td>
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<tr>
<td>8.69 mL BME, 100 µL 100X GlutaMAX, 200 µL 1.25 M HEPES, 10 µL 50 mg/mL Gentamicin, 1 mL AB+ Serum</td>
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<tr>
<td>Dulbecco's Modified Eagle Media (DMEM)</td>
</tr>
<tr>
<td>High Glucose, L-Glutamine, Gibco 11965-092</td>
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<tr>
<td>8.79 mL DMEM, 200 µL 1.25 M HEPES, 10 µL 50 mg/mL Gentamicin, 1 mL AB+ Serum</td>
</tr>
<tr>
<td>DMEM/F12, GlutaMAX, Gibco 10565-018</td>
</tr>
<tr>
<td>8.79 mL DMEM/F12, 200 µL 1.25 M HEPES, 10 µL 50 mg/mL Gentamicin, 1 mL AB+ Serum</td>
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<tr>
<td>Ham's Nutrient Mixture F12, GlutaMAX</td>
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<tr>
<td>Gibco 31765-035</td>
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<tr>
<td>8.79 mL F12, 200 µL 1.25 M HEPES, 10 µL 50 mg/mL Gentamicin, 1 mL AB+ Serum</td>
</tr>
<tr>
<td>M199, L-glutamine, Corning 10-060-CV</td>
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<tr>
<td>8.79 mL M199, 200 µL 1.25 M HEPES, 10 µL 50 mg/mL Gentamicin, 1 mL AB+ Serum</td>
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<tr>
<td>McCoy's 5A Media, High Glucose, GlutaMAX</td>
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<td>Gibco 36600-021</td>
</tr>
<tr>
<td>8.79 mL McCoy's 5A, 200 µL 1.25 M HEPES, 10 µL 50 mg/mL Gentamicin, 1 mL AB+ Serum</td>
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<tr>
<td>Modified Eagle Media (MEM), GlutaMAX</td>
</tr>
<tr>
<td>Gibco 41090-036</td>
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<tr>
<td>8.79 mL MEM, 200 µL 1.25 M HEPES, 10 µL 50 mg/mL Gentamicin, 1 mL AB+ Serum</td>
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<tr>
<td>Modified Eagle Media Alpha (MEMalpha), GlutaMAX</td>
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<tr>
<td>Gibco 32571-036</td>
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<tr>
<td>8.79 mL MEMalpha, 200 µL 1.25 M HEPES, 10 µL 50 mg/mL Gentamicin, 1 mL AB+ Serum</td>
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<td>Waymouth's Medium, L-Glutamine</td>
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<td>Gibco 11220-035</td>
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<td>8.79 mL Waymouth's, 200 µL 1.25 M HEPES, 10 µL 50 mg/mL Gentamicin, 1 mL AB+ Serum</td>
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<td>Ham's Nutrient Mixture F10, HEPES, L-Glutamine</td>
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<td>Gibco 12390-035</td>
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<td>8.99 mL F10, 10 µL 50 mg/mL Gentamicin, 1 mL AB+ Serum</td>
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<td>Iscove's Modified Dulbecco's Medium (IMDM), GlutaMAX</td>
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<td>Gibco 31980-030</td>
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<tr>
<td>8.99 mL IMDM, 10 µL 50 mg/mL Gentamicin, 1 mL AB+ Serum</td>
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<td>Roswell Park Memorial Institute Medium (RPMI), GlutaMAX, HEPES</td>
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<td>Gibco 72400-047</td>
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<td>8.99 mL RPMI, 10 µL 50 mg/mL Gentamicin, 1 mL AB+ Serum</td>
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<td>StemPro 34, SFM, Serum Free</td>
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<td>Gibco 10640-019</td>
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<tr>
<td>9.64 mL StemPro 34, 250 µL StemPro 34 Supplement, 100 µL 100X GlutaMAX, 10 µL 50 mg/mL Gentamicin, 1 mL AB+ Serum</td>
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<td>Hematopoietic Growth Medium (HPGM), Serum Free</td>
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<td>9.99 mL HPGM, 10 µL 50 mg/mL Gentamicin, 1 mL AB+ Serum</td>
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<tr>
<td>Osteoblast Basal Medium (OBM), 10% Serum</td>
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<td>Lonza CC-3208</td>
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<tr>
<td>9.99 mL OBM, 10 µL 50 mg/mL Gentamicin, 1 mL AB+ Serum</td>
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<tr>
<td>Stemline II Hematopoietic Stem Cell Expansion Medium, L-glutamine, Serum-Free</td>
</tr>
<tr>
<td>Sigma 50192-500mL</td>
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<td>9.99 mL Stemline II, 10 µL 50 mg/mL Gentamicin, 1 mL AB+ Serum</td>
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<tr>
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<tr>
<td>StemCell 09805</td>
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<td>9.99 mL Stemspan ACF, 10 µL 50 mg/mL Gentamicin</td>
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<td>Stemspan H3000, Serum Free</td>
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<td>StemCell 09800</td>
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<tr>
<td>9.99 mL Stemspan H3000, 10 µL 50 mg/mL Gentamicin</td>
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<tr>
<td>MarrowMAX, Bone Marrow Medium, Gentamicin, L-glutamine, FBS, Serum-Free</td>
</tr>
<tr>
<td>Gibco 12260-014</td>
</tr>
<tr>
<td>10 mL MarrowMAX Bone Marrow Medium</td>
</tr>
</tbody>
</table>

**Table 2.1** Culture media formulations for media screens.
2.3.5 Hypoxanthine Metabolic Labeling

We performed $^3$H-hypoxanthine uptake assays as described elsewhere for *P. falciparum* with some modifications [14]. All samples were prepared in 96-well flat bottom plates at a culture volume of 100 μL, 1% hematocrit, and starting parasitemia of ~2% or less, in IMDM or McCoy’s 5A. Cultures were supplemented after 40 hours maturation (unless otherwise noted) with 2.5 μCi $^3$H-hypoxanthine monochloride (PerkinElmer; Waltham, MA) in 0.2 culture volumes prepared in respective media. 2.5 μCi was determined to be an optimal amount of radiation, providing maximal signal while minimizing cost. We froze cultures at -80°C after a total of 44 hours maturation in standard culture conditions. After ≥24 hours, cultures were thawed, radioactive biomass was collected on 96-well UniFilter® GF/B plates (PerkinElmer; Waltham, MA) and quantified using a TopCount NXT or 1450 MicroBeta® TriLux liquid scintillation counter (PerkinElmer; Waltham, MA).
2.4 Results

2.4.1 *P. vivax* enrichment using small volume isolates

The low parasitemias characteristic of *P. vivax* infections severely limit the sensitivity of *ex vivo* assays. To robustly enrich the parasitemias of *P. vivax* human patient isolates, a KCl Percoll density gradient reticulocyte enrichment protocol was adapted [10]. Applying this enrichment technique to *P. vivax*-infected packed red blood cell (RBC) volumes as low as 100 µL, we observe significant enrichment of parasitemias when initial parasitemias are high enough to be visible by microscopy, or above approximately 0.005% (*Figure 2.1A*). This method enables robust *P. vivax* parasite enrichment ranging from approximately 10- to 100-fold, with 50% of tested isolates enriched > 30-fold and is functional with isolates from distinct geographical locations (India and Brazil), as well as isolates that are fresh or cryopreserved (*Figure 2.1B*). Furthermore, because only early-stage parasites typically survive the cryopreservation process [15], applying this enrichment method to cryopreserved isolates results in enriched ring stage *P. vivax*, whereas a mixture of parasite stages are enriched from fresh isolates (*Figure 2.1C*).
Figure 2.1 | *P. vivax* infected red cells can be reliably enriched from fresh or cryopreserved small-volume *ex vivo* human isolates from various geographical regions. A) Initial and enriched (post 1.080 g/mL KCl Percoll) parasitemia of *P. vivax* isolates (n = 20; p-value by paired Student’s t-test). B) Fold parasitemia enrichment of fresh and cryopreserved *P. vivax* isolates from either Brazil or India (n = 10 each location; error bars = Std. Dev.). C) Color-coded staging of parasite-enriched fractions after 1.080 g/mL KCl Percoll enrichment of fresh (average of n = 9) or cryopreserved (average of n = 9) *P. vivax* isolates with representative images of *P. vivax* staging (scale bars = 5 µM).
2.4.2 Optimization of *P. vivax* culture parameters

The sensitivity and reproducibility of *ex vivo* *P. vivax* assays are also hindered by often greater than 80% loss of parasite survival during a 44-hour ring to schizont *ex vivo* maturation course using standard protocols [16]. To enhance *P. vivax* *ex vivo* survival, we screened twenty different culture media to identify a formulation that maximized *P. vivax* survival (*Table 2.1*). We found Iscove’s Modified Dulbecco’s Medium (IMDM) significantly enhances the survival of human *ex vivo* *P. vivax* isolates through both asexual and gametocyte maturation over 44 hours of culture (*Figure 2.2A*). When compared to McCoy’s 5A, the current *P. vivax* culture media standard, IMDM improved the survival of both asexual as well as gametocyte stages for the vast majority of patient isolates tested (*Figure 2.2B*). Additionally, *P. vivax* parasites matured in either IMDM or McCoy’s 5A progress through asexual stages at similar rates (*Figure 2.2C*). Using IMDM as the base culture media, the additional parameters of ambient oxygen tension, serum supplement and hematocrit were varied to inform our standard culture parameters (*Figure 2.3*). Additionally, in order to identify the components of IMDM important for *P. vivax* survival enhancement, many attempts were made to supplement McCoy’s 5A with various IMDM nutrients, but minimal impact on the parasite survival was observed (*Figure 2.4 and Table 2.3*).
Figure 2.2 | IMDM enhances the ex vivo survival of asexual and gametocyte stages of *P. vivax* through intraerythrocytic maturation. A) Media screens for survival of asexual parasites or gametocytes through 36-44hrs ex vivo intraerythrocytic maturation (n = 3; multiplicity adjusted p-values *p < 0.0332, **p < 0.0021 by Dunnett's multiple comparisons test between IMDM and indicated media; error bars = SEM). B) Comparison of asexual parasitemia and gametocytemia in IMDM and McCoy’s 5A after 36-44 hours maturation (n = 11; p-values by paired Student’s t-test). C) Average progression of *P. vivax* asexual staging as measured by microscopy every 4-8 hours in IMDM and McCoy’s 5A (n = 5).
Figure 2.3 | Optimization of general *P. vivax* ex vivo culture parameters. The average parasitemias of *P. vivax* isolates were determined by microscopy after 44 hours post-thaw maturation in IMDM and the default culture parameters of 10% oxygen, 1% hematocrit and 10% serum, while varying the indicated culture parameters (n = 3 each panel, error bars = SEM).
Figure 2.4 | Supplementation of McCoy’s 5A and other Media with IMDM Nutrients. A-C) *P. vivax* was matured for 44 hours in IMDM, McCoy’s 5A or McCoy’s 5A plus the indicated supplement or mixtures of supplements at concentrations noted in Table S3. for A) 52 hours or B&C) 44 hours, and parasitemia and staging were performed via microscopy (n = 1 isolate each panel, error bars=range of parasitemia of technical duplicates). D) Sodium selenite was added to IMDM, McCoy’s 5A, RPMI and DMEM at 0.1X, 1X, 10X the typical IMDM concentration, and *P. vivax* was matured in each for 44 hours (n = 1, error bars = range of parasitemia of technical duplicates).
### Table 2.3 | Media Supplement Concentrations

<table>
<thead>
<tr>
<th>Inorganic Salts</th>
<th>Concentration (mM)</th>
<th>Amino Acids</th>
<th>Concentration (mM)</th>
</tr>
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<tbody>
<tr>
<td>Sodium Bicarbonate</td>
<td>9.8E+00</td>
<td>L-Alanyl-L-Glutamine</td>
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<td>Calcium Chloride</td>
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<td>L-Valine</td>
<td>6.5E-01</td>
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<tr>
<td>Potassium Nitrate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.5E-04</td>
<td>L-Isoleucine</td>
<td>5.0E-01</td>
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<sup>a</sup>These supplements were combined in the indicated concentrations to make “Supplement Mixture”

<sup>b</sup>This is the “1X” concentration where indicated
Compared to other tested media, including various published formulations of McCoy’s 5A (Table 2.2), *P. vivax* parasites in IMDM are more protected from death and hemolysis during the Stage I to Stage II transition as well as during the Stage II to Stage III transition (Figure 2.5A), which occur between 0 and 8 hours post-thaw and 20 and 28 hours post-thaw, respectively (Figure 2.5B). Nonetheless, there remains an approximately 50% decrease of viable *P. vivax* parasites in IMDM, but attempts to further enhance *P. vivax* survival by supplementing IMDM with various nutrients and additives have been largely unsuccessful (Figure 2.6 and Table 2.4).
Figure 2.5 | IMDM protects *P. vivax* infected RBCs from lysis early in intraerythrocytic development. A) Staging progressions (as represented in Figure 1) and parasitemias of *P. vivax* ex vivo cultures were assessed every 4-8 hours by microscopy and normalized to parasitemia at 0 hours post-thaw (n = 5). B) Parasite survival was followed over 44 hours of intraerythrocytic maturation in indicated culture media (Table S2), and non-dead parasitemia in each media was normalized to t = 0 hours post-thaw (n = 5, error bars = SEM).
Figure 2.6 | Supplementation of IMDM with Various Nutrients. *P. vivax* was matured for 44 hours in IMDM or IMDM plus the indicated supplement at concentrations noted in Table S4, and parasitemia and staging were assessed via microscopy (n = 1, error bars = range of parasitemia of technical duplicates).
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<td>Reduced Glutathione</td>
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<td>Antioxidant Supplement</td>
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2.4.3 Establishment of a rapid, highly-sensitive, $^3$H-Hypoxanthine *P. vivax* maturation assay

The current standard for assessing *ex vivo* *P. vivax* growth and maturation as well as drug sensitivity assays is a laborious and potentially subjective modified microscopy-based WHO microtest [9]. To rapidly and robustly measure IMDM-supported *P. vivax* survival through 44 hours of post-thaw maturation to schizogony, $^3$H-hypoxanthine metabolic labeling protocols routinely applied to *P. falciparum* were adapted [14]. A serial dilution of parasitemia revealed the superior sensitivity of this assay to detect parasite maturation in IMDM compared to McCoy’s 5A when $^3$H-hypoxanthine was added at 40 hours post-thaw (Figure 2.7A). Indeed, in the IMDM condition, the signal to noise ratio from parasites cultured without chloroquine was distinguishable over chloroquine-killed *P. vivax* with starting parasitemias even below 0.05% (Figure 2.7A). This assay can be utilized to generate antimalarial dose-response curves and calculate the inhibitory concentration at which 50% of parasites die ($IC_{50}$), and the fit of these curves to the data ($R^2$) is highly dependent on the post-enriched parasitemia at the initiation of the assay (Figure 2.7B). All tested isolates above 0.2% parasitemia, and most isolates between 0.045% and 0.2% parasitemia had a good fit ($R^2 \geq 0.9$), but under 0.045% parasitemia the fit was consistently poor ($R^2 < 0.9$) (Figure 2.7B). The fit of the $IC_{50}$ curve to the data is also influenced by the time at which $^3$H-hypoxanthine is added to the culture (Figure 2.7C). When $^3$H-hypoxanthine is added at 40 hours post-thaw and the fit is below $R^2=0.9$, adding $^3$H-hypoxanthine earlier in the 44-hour culture period can increase signal, producing a better fit as seen with isolates ECS, RCS and MCO (Figure 2.7C). However, if the curve fit is already above $R^2=0.9$ when $^3$H-hypoxanthine is added at 40 hours post-
thaw, adding it earlier does not significantly boost the fit as demonstrated by isolates MPL and ERS. Conversely, some isolates, like FBCS, have such a low parasitemia as to result in a poor fit independent of the time of addition of $^3$H-hypoxanthine.
Figure 2.7 | ^3H-hypoxanthine metabolic labeling of IMDM-supported *P. vivax* is an adaptable way to sensitively and rapidly measure *ex vivo* maturation. A) Representative charts of the effect of serial dilutions of initial parasitemia of a single *P. vivax* isolate on the metabolic labeling of chloroquine-treated or untreated parasites matured for 40 hours in IMDM or McCoy’s 5A, then isotopically labeled with 2.5 µCi/well of ^3H-Hypoxanthine for 4 additional hours (error bars = SD of technical duplicates). B) Effect of initial parasitemia on the fit ($R^2$) of the dose-response curves (IC$_{50}$) (Red/dark grey = poor fit, yellow/light grey = intermediate fit, green/white = good fit). C) Effect of the time of ^3H-hypoxanthine of addition on the fit ($R^2$) of the dose-response curves (IC$_{50}$) during a 44 hour culture of various isolates ($n = 1$ for each isolate).
2.4.4 Rapid surveillance of *P. vivax* chloroquine resistance and assessment sensitivity to lead antimalarial compounds

To define a baseline range of chloroquine sensitivity for the isolates collected in Brazil, the $^3$H-hypoxanthine metabolic labeling maturation assay was applied to isolates donated by 11 different individuals (Figure 2.8A). We found chloroquine IC$_{50}$ values against *P. vivax* range from approximately 9 to 23 nM, with an average and standard deviation of 17.4 ± 4.42 nM (Figure 2.8A). Also, we find the IC$_{50}$ values are highly reproducible across multiple vials from the same patient donation (Figure 2.9). Furthermore, to assess the sensitivity of our Brazilian isolates to various lead antimalarials from a range of classes and mechanisms of action, this assay was applied to isolates treated with DSM265, DSM421, KAE609 and GNF179, resulting in IC$_{50}$ values (95% CI) of 529 nM (375-746), 90.5 nM (65.1-128), 0.732 nM (0.474-1.13) and 1.88 nM (1.42-2.51) respectively (Figure 2.8B) [17, 18].
Figure 2.8 | Isotopic metabolic labeling of *P. vivax* facilitates rapid assessment of antimalarial sensitivities. Separate, Brazilian *P. vivax* cryopreserved human isolates were enriched on with 1.080 g/mL KCl Percoll and matured in IMDM for 40 hours, then labeled with 2.5 µCi 3H-hypoxanthine for 4 additional hours in technical duplicate. A) Individual dose-response curves with chloroquine concentrations diluted 2-fold from 400 nM to 3.125 nM, and a box and whisker plot of the calculated IC₅₀ values (n = 11). B) Dose-response curves of various lead antimalarial compounds (DSM265 and DSM421 n = 3, error bars = SEM, KAE609 and GNF179 n = 2, error bars = range).
Figure 2.9 | Reproducibility of chloroquine sensitivity across multiple replicates of two patient donations. Calculated IC$_{50}$ values of two biological replicates of each of two Brazilian *P. vivax* cryopreserved human isolates (MPL and ERS) enriched on with 1.080g/mL KCl Percoll and matured in IMDM for 40 hours, then labeled with 2.5µCi 3H-hypoxanthine for 4 additional hours in technical duplicate (n = 2, error bars = Std. Dev.).
2.5 Discussion

Drug resistance surveillance, development of novel antimalarials, and elucidation of basic *P. vivax* biology is currently limited by the various challenges of using *P. vivax* human isolates. This study reports methodologies that enhance the utility of cryopreserved *P. vivax* isolates from human patients in ex *vivo* maturation assays to permit rapid and robust measurements of drug resistance and susceptibility to novel antimalarials.

The use of Percoll density gradients for the enrichment of *P. vivax* ring parasitemias has been reported previously; however, these gradients are recommended for use on isolates with greater than 1mL of packed RBCs and result in an enrichment of approximately 10-fold [8]. Using Percoll dilution buffers with elevated KCl concentrations minimizes dehydration of the cells applied to the gradient, thereby increasing the magnitude and reproducibility of reticulocyte enrichment [10]. As *P. vivax* preferentially invades reticulocytes [19], this method results in an approximately 50-fold average enrichment of parasites from both fresh and cryopreserved isolates obtained from two distinct geographic regions (Brazil and India). Additionally, we find little parasite material in the enrichment gradient pellets, indicating a high percent yield of input parasites. We have successfully applied this method to packed cell volumes as low as 100 µL, which is approximately 10-fold less than previously recommended [8], enabling the use of small-volume cryopreserved isolates and the re-enrichment of small volume *P. vivax* cultures after *in vitro* reinvasion. Importantly, having enriched parasitemias allows a researcher to dilute the sample to a standard parasitemia to avoid the inoculum effect described when testing some antimalarials [20].
This enrichment technique is especially useful when applied to cryopreserved isolates, because only young ring forms typically survive the freeze-thaw process [15], effectively synchronizes cultures, facilitating downstream measurements of maturation. Furthermore, the use of cryopreserved *P. vivax* isolates permits the analysis of multiple isolates in batch, which minimizes assay-to-assay variability by enabling the comparison of antimalarial sensitivities of parasites from various geographical locations or different points in time within the same assay.

Many culture media formulations have been reported for *ex vivo* maturation of *P. vivax* [4, 7, 21, 22]. Most current formulations are based on McCoy’s 5A with human serum added with or without additional supplements. Unfortunately, there is a major loss of viable *P. vivax* parasites over 48hrs of a single round of intraerythrocytic maturation using McCoy’s 5A-based formulations [16]. Here, we show that IMDM enhances survival of all intraerythrocytic forms through a single round of maturation when compared to other media formulations. Furthermore, IMDM appears to protect against hemolysis of infected red blood cells (iRBCs) during the initial stages of maturation, as even iRBCs with “dead” parasites tend to remain intact and quantifiable for longer. Nonetheless, even with enhanced parasite survival with IMDM, the bulk *P. vivax* parasite density does decline over time, though the kinetics of stage development of the remaining iRBCs is largely normal. This reduction due to cellular lysis, together with reinvasion and subsequent survival remain major obstacles to sustained *in vitro* blood-stage *P. vivax* culture.

The use of $^3$H-hypoxanthine metabolic labeling to assess *Plasmodium* maturation has numerous advantages compared to other techniques, including rapidity, technical simplicity and objectivity, low background signal and high sensitivity. Indeed, even when
adding $^3$H-hypoxanthine as late as 40 hours post-thaw, we can achieve distinguishable signal over background after 44 hours of maturation with initial parasitemias below 0.05%. By adding $^3$H-hypoxanthine earlier, we boosted this sensitivity even more. Although, it should be noted that it is essential to consider the stage-specificity of drug action when deciding when to add $^3$H-hypoxanthine, as it is ideal to add the radiolabel after the drug elicits maximum killing. Because chloroquine acts early in the *P. vivax* life cycle [23], it is possible to add $^3$H-hypoxanthine as early as 24 hours post-thaw, boosting assay sensitivity. However, for antimalarials that act later in the parasite life cycle, and for antimalarials with an unknown timing of action, addition of $^3$H-hypoxanthine at 40 hours post-thaw maximizes the window for drug action to occur while still offering a sensitive readout.

Using this method, from a single cryopreserved isolate containing 0.5 mL packed blood, we regularly assay 20 conditions and have assayed up to 120 conditions in a 96-well plate format, although this is dependent on the specific patient isolate. Overall, when adding $^3$H-hypoxanthine at 40 hours post-thaw, we find that approximately one-third of tested Brazilian isolates provided high enough signal to generate a dose-response curve with a good fit, and this proportion of usable isolates could be boosted significantly by adding $^3$H-hypoxanthine earlier.

With chloroquine being the first-line treatment for blood-stage *P. vivax* infection in most endemic regions, and with resistance reported in nearly every region, it is now important to be able to rapidly and robustly monitor resistance to inform treatment expectations [3, 24]. Indeed, using our assay, we quickly determined the chloroquine sensitivities of 11 *P. vivax* isolates from Brazil, finding no resistant parasites (categorized
by an IC$_{50}$ > 100 nM) [25]. This is expected considering the estimated post-chloroquine treatment $P.$ vivax recrudescence rate in Brazil is between 2% and 10% [26]. Furthermore, using the isotopic metabolic assay our range of cryopreserved Brazilian $P.$ vivax chloroquine sensitivities is comparable to those reported using other assays with fresh $P.$ vivax from patients in Southeast Asia [27]. While the use of radiation may not be possible in many field sites, utilizing our improved techniques on cryopreserved isolates shipped to radiation-capable labs will enable researchers to quickly and simultaneously assess the antimalarial sensitivities of multiple $P.$ vivax isolates from different patients, geographic locations and/or points in time, at central and appropriately equipped laboratories.

In addition to monitoring chloroquine resistance, it is necessary to pursue novel $P.$ vivax chemotherapeutics. Our techniques can be applied to antimalarials from various drug classes. DSM265, the first dihydroorotate dehydrogenase (DHODH) inhibitor to reach clinical trials as a treatment for $P.$ falciparum, has approximately 5-fold better activity against $P.$ falciparum compared to $P.$ vivax while DSM421, another DHODH inhibitor, was developed to have improved activity against $P.$ vivax [17, 28], which is supported by our results. We also tested the sensitivities of Brazilian isolates to KAE609, a spiroindolone compound currently in clinical trials with $P.$ falciparum resistance-associated mutations accumulated in a P-type cation-transporter, PfATPase4, and we found activity similar to what has been shown using other assays [29, 30]. Finally, GNF179 is an imidazolopiperazine class compound, closely related to the promising KAF156 antimalarial, which elicits $P.$ falciparum resistance-associated mutations accumulated in cyclic amine resistance locus, pfcarl [31, 32]. We used our methods to
report the first activity of GNF179 against live *P. vivax* parasites, resulting in low nanomolar activity. Together, these data demonstrate the applicability of our techniques across different drug classes and mechanisms of action.

In conclusion, combining a KCl Percoll density enrichment method with enhanced maturation using IMDM as the base culture medium, greatly enhances our ability to analyze *P. vivax* isolates for a number of biological and public health applications. We have shown that \(^3\)H-hypoxathine metabolic incorporation methods can be applied for a highly sensitive, robust and rapid assessment of *P. vivax* *ex vivo* maturation, and that this can be directly applied to increase the utility of cryopreserved human isolates in measuring antimalarial susceptibility. With *P. vivax* resistance to chloroquine a very real threat, these methods will be influential in facilitating resistance surveillance as well as expediting drug discovery.
2.6 Acknowledgements

We thank Dr. Elizabeth Winzeler (University of California San Diego) for providing GNF179. GR was funded by the Gilliam Fellowship for Advanced Study through the Howard Hughes Medical Institute. UK is a recipient of a Canadian Institutes of Health Research Postdoctoral Fellowship. The clinical trial that originated samples from Brazil was supported by the Ministry of Health of Brazil and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), grant 404067/2012-3. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication. The authors declare that they have no competing interests.
2.7 References


CHAPTER THREE

*Plasmodium vivax* transcriptional profiling of low input cryopreserved isolates through the intraerythrocytic development cycle

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¶These authors contributed equally to this work.

**Author Contributions:** G.W.R, M.A.C., and M.T.D designed and planned the experiments; G.W.R., M.A.C, and U.K. performed parasite culture and FACS isolation; J.M.G. performed preliminary data quality assurance; G.W.R. performed alignments, complete data quality analysis, differential gene expression analysis, and all other data analysis; B.M. facilitated interactions with the Broad Technology Institute for RNA extraction and sequencing; M.J.M. and M.U.F. organized and performed Brazilian sample collections; and G.W.R., M.A.C., and M.T.D. wrote the manuscript.
3.1 Abstract

Approximately one-third of the global population is at risk of *Plasmodium vivax* infection, and an estimated 7.51 million cases were reported in 2017. Although, *P. vivax* research is currently limited by the lack of a continuous *in vitro* culture system for this parasite, recent work optimizing short-term *ex vivo* culture of *P. vivax* from cryopreserved isolates has facilitated quantitative assays on synchronous parasites. Pairing this improved culture system with low-input Smart-seq2 RNAseq library preparation, we probed the transcriptional signature of *ex vivo* *P. vivax* samples from three different patients using only 1000 parasites per sample, generating transcriptomic data at different stages over the course of the intraerythrocytic development cycle (IDC). Using this strategy, we achieved similar quality to previously reported *P. vivax* transcriptomes. We found little effect with varying culture media on parasite transcriptional signatures, identified many novel gametocyte-specific genes from transcriptomes of FACS-isolated gametocytes, and determined invasion ligand expression in schizonts in biological isolates and across the IDC. In total, these data demonstrate the feasibility and utility of *P. vivax* RNAseq-based transcriptomic studies using minimal biomass input to maximize experimental capacity.
3.2 Introduction

Although malaria-causing parasites, including *P. falciparum* and *P. vivax*, were discovered nearly 130 years ago, and the blood stages of *P. falciparum*, the deadliest species of malaria parasite, has been culture-adapted since 1976, continuous *P. vivax* blood-stage culture remains elusive, significantly hindering experimentation and the development of new therapeutic strategies [1, 2]. This contributes to the fact that *Plasmodium vivax* remains a significant global health problem, putting approximately one-third of the global population at risk for infection and resulting in an estimated 7.51 million reported cases in 2017 [3]. Furthermore, relative to other malaria species, *P. vivax* has many unique and challenging biological qualities including the presence of spontaneously reactivating dormant liver stages (hypnozoites), strong preference for invading the youngest circulating host red blood cells (reticulocytes), and the rapid development of transmissible gametocytes often before clinical symptoms present [4].

Characterizing the transcriptomic profiles of various stages of this parasite has potential to yield important advancements in understanding the biology underlying these unique traits, thereby enabling many new experimental studies and public health interventions. Indeed, transcriptome profiles of *P. vivax* across the intraerythrocytic development cycle (IDC) have been generated using both microarray and RNAseq approaches, but because of the lack of a continuous *in vitro* culture system for the IDC of this parasite, these studies have been either limited to characterizing IDC stages immediately available from the patient [5-7], or have been limited to research groups that have access to both fresh, clinical isolates as well as proximal, well-equipped laboratory space for sample processing [8, 9]. Furthermore, while some of these publications report
the robust transcriptomic characterization of patient isolates of *P. vivax* through the 48-hour IDC, to date, these studies have required large amounts of fresh starting material (10-20 mL whole blood samples), and these study designs are not able to isolate transcriptomes of sexual-stage from asexual-stage intraerythrocytic parasites, a distinction that would greatly enable understanding the unique *P. vivax* gametocyte biology and the development of transmission-blocking interventions [8].

One reason a relatively large amount of starting material is required for these studies is the high rate of loss of *P. vivax* parasitemia through the course of the IDC when using McCoy’s 5A culture media [10, 11]. In recent work, we demonstrated that various culture media formulations have significant impact on the survival of *P. vivax*, and that the use of Iscove’s Modified Dulbecco’s Medium (IMDM) boosts ex vivo *P. vivax* survival for short-term culture, expanding the utility of small-volume, cryopreserved isolates [11]. Furthermore, recent advancements in RNA library preparation for RNAseq transcriptomic approaches have greatly improved detection, coverage, bias and accuracy for relatively small amounts of input material [12].

Another hurdle in generating robust transcriptional data from patient samples is the low fraction of parasite RNA in a samples relative to human RNA, with parasite transcripts often accounting for far less than half of sequenced RNA [5, 7, 9]. This is likely a result of the presence of host leukocytes that are collected along with the parasitized erythrocytes during patient sample acquisition. Fortunately, techniques for both filtering away leukocytes from patient samples as well as purifying *Plasmodium* parasites via fluorescence-activated cell sorting (FACS) have been established [13, 14], and employing these may reduce the impact of this hurdle on acquiring strong transcriptomic data.
An additional challenge in studying the transcriptomes of various stages of the *P. vivax* IDC is the asynchrony of parasites in a patient sample. Indeed, it has been reported that the vast majority of transcripts characterized from parasites directly from blood-draw are from trophozoites [5, 7]. This is likely because later stage parasites tend to have more transcript abundance, and because the presence of later stages of *P. vivax* parasites is much less likely in circulating blood compared to earlier stages [15]. However, as we reported previously, only the early IDC stages tend to survive our reported cryopreservation and thawing process, thereby facilitating the study of relatively synchronous parasite populations [11].

This present study capitalizes on these advancements in the culture of viable cryopreserved *P. vivax*, RNAseq library preparation and parasite isolation to generate robust transcriptomes of *P. vivax* from just 1000, *P. vivax*-infected erythrocytes purified by FACS at various time points throughout the IDC from three separate cryopreserved clinical isolates [11, 12].
3.3 Materials and Methods

3.3.1 Ethical Approval and Sample Collection

All study subjects provided written, informed consent. The Institutional Review Board of the Institute of Biomedical Sciences at the University of São Paulo, Brazil approved the protocols for parasite sample collection (1169/CEPSH, 2014). Samples were collected in Mâncio Lima, Acre State, Brazil, from *P. vivax* patients diagnosed via conventional thick-smear microscopy performed in the context of a randomized, open-label clinical trial [16], and the samples were depleted of leukocytes and cryopreserved as previously described [17].

3.3.2 *P. vivax* Maturation and RNA Sample Collection

We prescreened approximately 15 cryopreserved, leukofiltered blood isolates from separate Brazilian donors for acceptable recoverable parasitemia (>0.2% post enrichment). Vials from three of the acceptable donations were thawed and enriched on a 1.080 g/mL KCl Percoll gradient as previously described [11], the enriched parasites were plated at 10E7 cells per mL (~1% hematocrit) in indicated culture media supplemented with 10% v/v AB+ heat-inactivated human serum pooled from 15 human donors and 50 µg/mL gentamicin. Samples were collected at indicated times for FACS and microscopy (Figure 3.1A). For each technical replicate, the sample was stained with Vybrant DyeCycle Green (1:5000) for 20 minutes at 37°C, then 1000 individual parasitized cells were sorted into lysis solution made of 1x Buffer TCL (Qiagen) with 1% v/v 2-mercaptoethanol. Sorting was performed on a BioRad S3e sorter or Sony SH800 sorter. *P. vivax* infected cells were selected by gating for single events then for DNA+ cells.
(Figure 3.1B), with additional cells sorted for microscopy slides when remaining biomass permitted. Upon collection, samples were immediately placed on dry ice and then transferred to -80°C storage. Once samples from all time points were collected, samples were thawed and plated onto either an RNA-low or RNA-high plate based on expected sample RNA content as indicated (Figure 3.1A) and submitted to the Broad Technology Labs for library preparation via the Smart-seq2 strategy using 18 cycles of PCR amplification [12]. Sequencing, which was performed on the Illumina NextSeq500 platform using High Output v2 kit. Reproducibility across flow cells was verified, and the data was concatenated before additional processing.
Figure 3.1 | Small volume, cryopreserved *Plasmodium vivax* isolates provide robust RNAseq data via the Smart-seq2 platform. A) Experimental strategy. Three separate Brazilian *P. vivax* patient isolates were thawed, enriched, and cultured in four different culture media. Samples were taken at 4, 20, 36, 44 and 72 hours post-thaw for FACS, provided enough biomass remained, and were sorted based on DNA content or DNA high versus DNA-low when applicable. B) The FACS gating scheme used to separate infected RBCs from uninfected RBCs using Vybrant DyeCycle Green stain. C) Quality control analysis of each individual sample. The hashed horizontal lines represent previously established thresholds below which any particular sample would have been excluded. Error bars represent the standard deviation of technical replicates. D) Transcriptome saturation curves for each sequencing plate showing % protein-coding genes covered (TPM > 1) when 10%, 25%, 50%, 75%, 90%, and 100% of generated reads were analyzed.
3.3.3 Read Alignment, Quantification and Analysis

Paired-end reads were aligned to the *Plasmodium vivax* P01 genome [18] using Spliced Transcripts Alignment to a Reference (STAR) v2.5.0c [19] with the parameters -runThreadN 16, --runMode alignReads, --twopassMode Basic, --alignIntronMax 500, --alignMatesGapMax 500, --sjdbScore 2, --quantMode TranscriptomeSAM GeneCounts, -outReadsUnmapped Fastx, and --sjdbOverhang 24. Reads were quantified and transcripts per million were calculated using the RNAseq by Expectation-Maximization (RSEM) package v1.2.29 [20] with the command and parameters rsem-calculate-expression -p 16, paired-end, --bam, and --output-genome-bam. Picard Tools v2.18.7 was used to assess library size and percent high quality mapped reads. Differential gene expression analysis was performed using the Empirical Analysis of Digital Gene Expression Data in R (edgeR) package v3.20.9 [21]. Enriched gene ontology term tree maps of biological processes corresponding to schizont and gametocyte populations were created using REVIGO analysis of all genes significantly (Benjamini-Hochberg adjusted p-value < 0.05) upregulated in each population by at least log₂(fold change) > 2 [22]. Principal components analysis was performed through the *prcomp* function in the *stats* v3.4.4 package of RStudio v 1.1.447 on log₂(TPM+1) data generated via RSEM.
3.4 Results

3.4.1 Quality Assurance

To ensure the quality of these data, various metrics were assessed for every sample, and each passed thresholds previously established for Smart-seq2 RNAseq libraries generated from single mammalian cells [23], including library size > 1 million pass-filter (PF) high-quality (HQ) reads, > 70 percent HQ reads mapped to the target genome (*Plasmodium vivax* P01) [18], and > 35 percent of protein coding genome covered above an empirically chosen threshold of gene expression, transcripts per million (TPM) ≥ 1 (*Figure 3.1C and 3.2*). Notably, each of our sample libraries consisted of reads of which 89%-96% (median 94%) mapped to the *PvP01* genome (*Figure 3.1C*). Furthermore, to assess how comprehensively the data represent the transcriptome of each sample, transcriptome saturation curves were created, and the percentage of protein-coding genes with TPM ≥ 1 for every sample largely plateaus as total reads analyzed approaches 100% (*Figure 3.1D*).
Figure 3.2 | A threshold of TPM = 1 for gene expression is empirically reasonable. Density curve of the calculated gene expression represented as $\log_2(TPM+0.1)$ across all technical and biological replicates with the vertical dotted red line representing the TPM = 1 threshold for categorizing a gene as expressed.
3.4.2 Culture Media Effects on *P. vivax* Transcriptomes

In previous work, we demonstrated significant effects of culture media on *P. vivax* ex vivo survival, and because the biological explanations for these effects remain elusive, we sought to identify a transcriptional signature that may help elucidate the mechanism of differential growth in various media formulations. When performing principal components (PC) analysis, approximately 60% of variation in transcriptional signatures across samples can be accounted for by the first two principal components, which account for 44.51% and 15.05% of sample variation respectively (Figure 3.3). The primary source of sample separation on a scatter plot of PC1 and PC2 is explained by sample time point through the IDC, and secondary separation is evident between the three patient isolates (Figure 3.4). The separation between patient isolates is especially evident at the early stages of the IDC, where, for instance, the hour 4 post-thaw group has the widest spread, and there is overlap on the PC plot between isolate *PvMRMS* at hour 4 post-thaw and *PvJBC* at hour 20 post thaw (Figure 3.4). However, the general directional trend on the PC plot for each isolate through the IDC over time is consistent, and the transcriptional profiles tend to move toward convergence between isolates at the later developmental stages (Figure 3.4). Notably, there is negligible separation on the PC plot accounted for by culture media (Figure 3.4).
Figure 3.3 | The majority of transcriptional variation is explained by the first two principle components. A scree plot showing the proportion of variance in gene expression explained by each of the top ten principle components generated.
Figure 3.4 | The majority of transcriptional variation is explained by IDC stage and patient isolate. A scatter plot of the first two principle components with point colors and associated polygons representing different time points of samples analyzed, point shapes representing different media used, and point size representing the different patient isolate sampled. The hashed box highlights overlap between isolate \textit{PvMRMS} at hour 4 post-thaw and \textit{PvJBC} at hour 20 post thaw.
3.4.3 Transcriptome from Purified Gametocytes

As the parasites develop from rings to a mixture of schizonts and mature gametocytes, we find that distinct populations of parasites with various levels of DNA content emerge and can be distinguished by flow-cytometry (Figure 3.5). For one isolate (PvTCF), enough material was available to extend sampling to 72 hours post-thaw, enabling the fractionation of a sexual-stage population from an asexual-stage, schizont population via FACS (Figure 3.6A). The relative stage homogeneity of each sort was verified by plotting the averaged expression across all biological replicates matured in IMDM of known/putative sexual-stage and schizont-stage genes at each time point and sorted population on a heatmap [24, 25], clearly showing the 72-hour DNA-mid and DNA-low populations represent a primarily sexual transcriptomic profile, while the 72-hour DNA-high sort represents a primarily schizont transcriptomic profile, exclusively (Figure 3.6B). Also, there is strong agreement between our P. vivax gametocyte transcriptome and the previously published gamete/zygote transcriptomes, with 12 of their 25 most highly expressed gamete/zygote genes appearing in our top 100 genes most differentially expressed in gametocytes versus schizonts [6].
Figure 3.5 | Flow cytometry and representative microscopy image of P. vivax parasites throughout the intraerythrocytic development cycle. (A) Flow cytometric plots of gate-purified P. vivax (Fig. 1B) comparing DNA content by Vybrant DyeCycle Green stain and side scatter at each sampled time point for isolate PvTCF grown in IMDM. (B) Representative images of P. vivax parasites from each time point. Black bars represent 10 μm.
A list was generated of genes, including many previously uncharacterized genes, that are selectively upregulated in either the gametocyte or schizont populations (Table 3.1). Some of the most highly expressed genes upregulated in *P. vivax* gametocytes, including P25 and Lap5, have already been confirmed as *P. vivax* gametocyte markers [26-28]. Furthermore, tree maps of biological processes corresponding to the gene ontology terms of significantly upregulated genes in the gametocyte and schizont populations were created, showing gametocytes transcripts were mostly devoted to nucleoside phosphate biosynthesis, while schizont transcripts correspond to processes related to interspecies interactions (Figure 3.6C and D).
Figure 3.6 | Sorting iRBCs by DNA content at 72 hours post-thaw enables isolation of gametocyte from schizont transcriptomes. A) The gating strategy for DNA-high, DNA-mid and DNA-low FACS-sorted populations after gating for parasite positive cells as in Figure 1. Representative images of parasites from each sorted population shown with black bar representing 10 micrometers. B) Heatmap depicting the expression in log2(TPM+1) averaged across all available biological replicates of known sexual stage and known schizont stage populations shown with black bar representing 10 micrometers. B) Heatmap depicting the expression in log2(TPM+1) averaged across all available biological replicates of known sexual stage and known schizont stage populations shown with black bar representing 10 micrometers. C & D) REVIGO tree maps of gene ontology term biological processes for all genes significantly (Benjamini-Hochberg adjusted p-value < 0.05) upregulated by at least log2(fold change) > 2 in the C) gametocyte population or D) the schizont population.
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FC = Fold Change; CPM = Counts-per-million as calculated by EdgeR; * p-values are adjusted by the Benjamini & Hochberg method.
3.4.4 Invasion Ligand Expression

Next, to investigate the variation in expression of various known or putative *P. vivax* erythrocyte invasion ligands and invasion ligand families in the schizont stages, the average TPM of each ligand was plotted for the 44-hour, DNA-high populations of isolates TCF and MRMS grown in IMDM (Figure 3.7). This shows a wide variation in invasion ligand expression within an isolate, ranging between 0 and 15000 TPM with ligands MSP1, MSP9, RAMA and RhopH3 among the highest expressed putatively functional [29] invasion ligands in both isolates (Figure 3.7A). Also, the general level of invasion ligand expression notably varies between the two isolates, especially among the RBP, MSP3 and TRAG ligand families (Figure 3.7B-D). Lastly, to temporally characterize the expression of *P. vivax* invasion ligands, the average TPM of each invasion ligand plotted on a heat map for every time point over the intraerythrocytic development cycle for each isolate grown in IMDM, showing that, as expected, the expression of most invasion ligands peaks in the DNA-high populations (Figure 3.8).
Figure 3.7 | Expression of *P. vivax* invasion ligands. Expression levels in transcripts per million (TPM) within isolates *PvTCF* and *PvMRMS* at 44 hours maturation in IMDM and FACS sorted by the DNA-high gate of A) confirmed and putatively functional invasion ligands, B) reticulocyte, Duffy, and erythrocyte binding protein (RBP, DBP, and EBP) families, C) merozoite surface protein 3 (MSP3) family and D) tryptophan-rich antigen (TRAG) family. Error bars represent the standard deviation between technical replicates.
Figure 3.8 | Expression of putatively functional *P. vivax* invasion ligands varies across intraerythrocytic development. Heatmap depicting the average invasion ligand expression in log2(TPM+1) by isolates grown in IMDM across the intraerythrocytic development cycle.
3.5 Discussion

Though *P. vivax* RNAseq transcriptomes through the IDC have been previously generated, these transcriptomes have relied upon clinical samples that can yield relatively large quantities of RNA. The finite nature of clinical samples paired with the characteristic low parasite densities and poor *in vitro* survival of *P. vivax*, severely limits the ability to include experimental perturbations in *P. vivax* transcriptome studies. This study describes the first use of viable *P. vivax* clinical isolates recovered from cryopreservation for the generation of robust transcriptomes using FACS-purified parasites at various stages of maturation throughout the IDC. By combining the Smart-seq2 RNAseq library preparation strategy for low-RNA samples with our recent advancements in *P. vivax* *ex vivo* culture from cryopreserved isolates, we achieve high-quality transcriptomes from just 1000 FACS-purified, infected erythrocytes per sample [11, 12]. In addition to establishing a novel *P. vivax* RNAseq strategy, we have 1) determined that culture media has relatively minimal effect on transcriptional signatures compared to parasite stage and clinical isolate, 2) generated the first transcriptome of purified *P. vivax* gametocytes and compared it with the transcriptome of purified *P. vivax* schizonts, and 3) examined *P. vivax* invasion ligand expression in schizont stages.

RNAseq provides a comprehensive snapshot of the transcriptomic environment of the subject, as opposed to microarray studies, which are inherently targeted. However, because of a lack of *in vitro* culture system for the intraerythrocytic stages of *P. vivax*, RNAseq studies for this parasite have been limited by many factors. First, finding or developing the combination of access to fresh clinical isolates and a proximal, well-equipped laboratory to process the samples is particularly challenging and resource-
intensive, especially as *P. vivax* is most prevalent in resource-poor settings. The use of cryopreserved clinical isolates removes the stipulation of having an experimental laboratory near the clinic; however, to-date no studies have used viable cryopreserved isolates for investigating transcriptomics throughout *P. vivax ex vivo* maturation. Indeed, there is only one report of RNAseq analysis over the course of the *P. vivax* IDC, and this required the use of relatively large volumes of whole-blood starting material, due to the historical difficulty of *P. vivax ex vivo* culture, where parasitemias notably decrease over the course of the IDC [9, 10, 30]. Though, several groups have recently improved enrichment strategies as well as the survival of *P. vivax* through *ex vivo* maturation, we are able to obtain and maintain a higher parasitemia over the IDC, allowing the use of much less starting material for experimental studies [11, 31].

An additional complication that often accompanies transcriptomic studies of *P. vivax* from whole blood samples is the prevalence of human transcripts in the RNA library, which can reduce the sensitivity of sequencing to parasite reads. 89-96% of our generated reads map directly to the *PvP01* genome, indicating a very low quantity of contaminating human RNA in our preparations. This is likely because our process includes an established, robust but simple leukodepletion method prior to cryopreservation [17], and because we FACS-purified infected erythrocytes by DNA content before RNA isolation. With total read numbers between 5 and 10 million mapped reads per sample, we get similar results to previous *P. vivax* RNAseq experiments using small-volume isolates [5], and our transcriptome saturation curves all plateau, indicating the dataset has captured the vast majority of genes expressed for each sample. Important to note is that excessive preamplification of the cDNA could create systematic bias in the
reads detected, and that while other *Plasmodium* transcriptomic studies have utilized up to 30 rounds of PCR preamplification, this study only used 18 cycles reducing the chance for bias [32, 33]. Another important contrast is that most previous *P. vivax* RNAseq studies have chosen to use the Salvador-I genome as a reference, but our use of the newer *PvP01* reference genome may assist in generating more robust data and mapping efficiency, because the *PvP01* genome is a nearly 10% larger assembly with much deeper fold-coverage and 22% more genes than Salvador-I, largely in the subtelomeric regions [18].

Although it is clear that culturing *P. vivax* in IMDM enables the parasites to survive much better than when cultured in formulaically similar Dulbecco’s Modified Eagle’s Medium (DMEM) or in the current *P. vivax* standard, McCoy’s 5A, the causal formulation components remain unclear [11]. We opted to test the effect of these various media as well as another hematopoietic cell culture medium, Aim V, on the transcriptomic profiles of intraerythrocytic *P. vivax*, with the goal of identifying gene expression responses that may elucidate the biological processes better supported by IMDM. The PC analysis does clearly show that the vast majority of variation in transcriptomes is due to parasite stage, which is expected as *Plasmodium spp.* transcriptional signatures are known to vary widely across the IDC [34]. Additionally, the secondary variation accounted for by clinical isolate, which is especially noticeable at early stages, is also anticipated for two reasons. First, it has been noted across many *Plasmodium* species that there is relatively large transcriptional variation between early stage parasites [34]. Second, although only the *P. vivax* parasites in the early stages of the IDC tend to survive our cryopreservation process [11], the isolates from separate patients are still likely to represent a range of early stage
parasites. Because these are primary patient isolates, it is impossible to control for the range of early stages present at the time of sample collection. Indeed, we find that there is marked overlap on this plot of the 4-hour PvMRMS samples with the 20-hour PvjBC samples, which suggests the parasites from the PvjBC isolate were much younger than the parasites from PvMRMS at thaw (Figure 3.4). Nonetheless, each isolate ultimately follows similar transcriptomic paths through the IDC, which is supported by the observation that the general trajectory of each biological replicate is similar in the two-dimensional PC analysis space as the IDC progresses (Figure 3.4). Remarkably, there is little separation in the PC analysis accounted for by culture media, and we found no significant, biologically reproducible, transcriptomic signature distinguishing parasites grown in IMDM versus to other media. Although they do not help define the mechanism of differential growth in various media formulations, these results may indicate a lack of transcriptional flexibility of P. vivax at each time point of the IDC, which could help explain the relatively fastidious nature of P. vivax ex vivo culture. Moreover, from our previous study, it is clear that when the parasites do die in each media, they are not visible as pyknotic cells, but rather disappear entirely, suggesting a red blood cell-focused hemolytic event [11]. Thus, the lack of transcriptional response to various media may indicate the media are directly affecting the host cell itself, resulting in either hemolysis or relative stability; however, additional studies are required to parse the role of the host cell in unsuccessful P. vivax in vitro maturation.

Previously, the transcriptomes of enriched P. vivax gametes, zygotes and ookinetes have been characterized through microarray analysis [6], but an RNAseq-based transcriptome of the purified intraerythrocytic P. vivax gametocytes has not been
achieved, again, largely due to the difficulty in generating sufficient gametocyte material for RNA library generation. Because we utilize viable *P. vivax* parasites recovered from cryopreservation, we are able to perform our experiments geographically separated from the largely resource-poor, endemic area from which the samples are obtained. This enabled us to capitalize on the state-of-the-art facilities and resources available to us, maximizing the viability of the parasites, even out to 72 hours post-thaw for one isolate, enabling the separation of enough gametocytes from schizonts to achieve robust transcriptomic information. It does remain unclear why there remain schizont parasites at this late time point. Possible explanations could be a large heterogeneity in the maturation time of asexual *P. vivax* parasites, or the potential stall of maturation and subsequent reactivation, a phenomenon which has been considered previously [30]. However, the 72-hour DNA-high parasites cluster very closely to the 44-hour DNA-high parasites on the PC plot, suggesting that these two populations are transcriptionally quite similar despite their temporal separation, providing confidence in the generation of a compressive list of genes that are selectively upregulated in gametocytes compared to schizonts. This list of putative *P. vivax* gametocyte markers has many proteins previously implicated in *Plasmodium spp.* gametocyte biology including G377, Lap5, and P25 [6, 26-28, 35]. Interestingly, this list also includes PSOP12, which has been shown to induce transmission blocking immunity in *P. berghei* mouse models, as well as PVP01_1252300, which has a *P. falciparum* ortholog that is a likely target for several Medicines for Malaria Venture Malaria Box compounds [36, 37]. Other, uncharacterized genes in this list could also be suitable antimalarial or vaccine targets in the future.
In terms of invasion ligand expression, though there is notable variation within an isolate, we find highly antigenic ligands, such as MSP1, MSP9, MSA180 and RAMA, are most highly expressed, which is encouraging because each of these is also being considered for vaccine development [38-40]. It is important to note, however, that while there are marked differences in invasion ligand expression between the isolates, an unknown portion of this variation is likely explained by the differences in the maturity of the schizonts, making it difficult to compare across biological replicates. Future studies should be performed with sufficient temporal sampling resolution to enable the employment of established statistical methods to accurately estimate the maturation state of the parasites [41]. Additionally, future RNAseq studies using many more isolates from geographically distinct regions would be beneficial in characterizing the full diversity of invasion ligand expression by the P. vivax parasite, especially among multigene families like the MSP3s, TRAGs or RBPs. Lastly, employing the growing ability of Plasmodium spp. single-cell transcriptomic approaches [32, 42] would be quite helpful in characterizing the variation of invasion ligand expression between parasites within an isolate, which would help determine whether such variation is more apparent between infections or between parasites within an infection.

In summary, this study provides strong evidence that small volume, cryopreserved P. vivax isolates can be used to attain robust RNAseq information, enabling the acquisition and analysis of transcriptomic information at research sites geographically removed from clinical sites of sample acquisition. Furthermore, this approach reduces experimental variability across biological replicates as transcriptomic studies from various cryopreserved P. vivax isolates can be performed in batch rather than sequentially as live
isolates present in the clinic. This study reports the FACS-based isolation of gametocytes from asexual parasites, allowing us to generate the first transcriptome from purified *P. vivax* gametocytes and to compare the expression of various invasion ligands in purified *P. vivax* schizonts. Utilizing purification methods, we recommend future studies generate schizont transcriptomes from many isolates to more robustly investigate the differential expression of invasion ligands between infections to better inform *P. vivax* vaccine development. Moreover, although we confirm large differences in transcriptome profiles between patient isolates, this is likely due to differences in the parasite staging at time of sample acquisition, which could be resolved in the future utilizing statistical methods on data with higher temporal resolution [41]. Such experiments could help to inform the development of novel vaccines, chemotherapeutics and experimental techniques. Indeed, this present study empowers such *P. vivax* transcriptomic experiments.
3.6 Acknowledgements

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3.7 References


CHAPTER FOUR

Elucidating the intraerythrocytic *Plasmodium vivax* niche among the heterogeneous reticulocyte population

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4.1 Abstract

One unique biological characteristic of Plasmodium vivax is the well-established restriction of the intraerythrocytic stages to young reticulocytes. However, short-term ex vivo studies by our group and others have revealed that the majority of P. vivax-infected cells lyse prematurely, even when the parasite is within young reticulocytes. Because reticulocytes are continuously maturing, they represent a dynamic and highly heterogeneous population with differences in surface protein expression, cytoskeleton composition, and overall metabolic capacity. Thus, viable P. vivax infection may be contingent on invading a particular reticulocyte subpopulation that could be defined by previously unrecognized surface markers. To identify novel markers of reticulocyte subsets, we performed quantitative surface proteomics of reticulocyte subpopulations representing various stages of development based on transferrin receptor (TfR/CD71) expression and RNA content the current standard markers for young reticulocytes. We detected 158 plasma membrane proteins of which 23 were lost rapidly during reticulocyte maturation, therefore representing the youngest reticulocyte subpopulations. We then screened a subset of these novel young reticulocyte markers for their presence on infected cells from cryopreserved Brazilian P. vivax isolates. We discovered that P. vivax parasites are approximately four times more likely than expected to be in a cell positive for one of our novel markers, SLC12A6, a potassium chloride cotransporter responsive to osmotic stress, than in cells positive for TfR/CD71. Furthermore, infected cells displaying this ion channel are more likely to support P. vivax survival through intraerythrocytic maturation, and his effect is abrogated when the channel is inactivated by treatment with a specific small molecule. Finally, comparing the P. vivax-supporting
IMDM culture media with less supportive culture media highlights a reduced concentration of major ion species, including sodium, chloride and potassium, in IMDM, and increasing the sodium chloride levels decreases *P. vivax* survival. Altogether, these results identify a subpopulation of reticulocytes designated by a potassium chloride cotransporter that is functional in supporting *P. vivax* intraerythrocytic growth and implicate the concentration of ions in the extracellular environment as critical to successful culture. These findings help define the preferred host cellular niche of intraerythrocytic *P. vivax* and could facilitate the establishment of robust continuous *in vitro* culture.
4.2 Introduction

*Plasmodium vivax* remains a significant threat to public health, with approximately one third of the global population at risk of infection with this malaria parasite [1]. Indeed, addressing this public health problem is particularly difficult for many reasons. For example, resistance to chloroquine, the frontline compound used to treat the symptom-causing blood stages of the parasite, has been reported in nearly every geographical region where *P. vivax* is endemic [2]. Also, this species of malaria parasite has a poorly understood stage called hypnozoite that remains undiagnosable in dormant liver stages until it reactivates and causes a relapse in blood-stage infection up to months or years after the primary infection. Furthermore, the only treatments available for this liver stage, primaquine and tafenoquine, are potentially deadly to people with glucose-6-phosphate dehydrogenase deficiency, a genetic polymorphism affecting up to 32% of people in some endemic regions [3, 4]. Finally, although there has been a culture system for the blood-stages of *P. falciparum* since 1976, there remains no robust continuous *in vitro* culture system for *P. vivax*. Because of this, the ability to apply experimental approaches toward things like drug target identification, large-scale compound screening, and vaccine development is greatly hindered.

Recent publications have addressed some of the main challenges of establishing *P. vivax in vitro* culture, including implementing reticulocyte isolation protocols to enhance enrichment of parasitemias from very low percentages found in donor circulation to workably high percentages, which facilitating quantification [5, 6]. Furthermore, *P. vivax* survival through the *ex vivo* intraerythrocytic development cycle (IDC) has been boosted by applying a particular media formulation, Iscove’s Modified Dulbecco’s Medium (IMDM),
enabling the ability to regularly achieve healthy schizonts in culture [5]. Taking a stepwise approach, the next challenge to overcome on the path toward continuous and robust in vitro culture is establishing reproducibly successful reinvasion of merozoites from ex vivo-matured schizonts into new host cells.

However, achieving robust P. vivax reinvasion is particularly difficult given the restrictive tropism of P. vivax for reticulocytes, the youngest forms of circulating erythrocytes [7, 8]. After the enucleation of erythropoietic precursors within the bone marrow, these cells egress into peripheral circulation as reticulocytes and continue to mature over the course of 12-48 hours [9]. This maturation process involves the loss of remaining organelles like the mitochondria and ribosomes, completion of protein translation, remodeling of the cytoskeleton, and the alteration (mostly reduction) of various surface proteins, including transferrin receptor (TfR or CD71) [10-14]. Because of these rapid and extreme changes during maturation, reticulocytes represent a highly dynamic and heterogeneous cell population with many potential subsets that may be more or less supportive of P. vivax invasion and subsequent maturation [15]. Indeed, it has been definitively shown that P. vivax preferentially invades a very young subpopulation of reticulocytes defined by high levels of residual ribosomes and RNA that make up the reticulum and high surface expression of CD71 [16-19]. However, it is also clear from various studies that reinvasion into this subset of reticulocytes and subsequent survival is not robust enough to support continuous and robust in vitro culture, which suggests there may be a different or more specific subset of host cells required by P. vivax [18, 20].

The present study aims to identify novel surface proteins that delineate novel reticulocyte subpopulations by utilizing surface proteomic characterization of circulating
reticulocytes at various stages of maturation. This enabled the discovery a reticulocyte subset that supports *P. vivax in vitro* maturation better than young reticulocytes defined by high CD71 expression. Furthermore, we implicate the functionality of the marker, a potassium chloride cotransporter, and the concentration of culture media ions as important for enhanced *P. vivax* survival through the IDC.

4.3 Materials and Methods

4.3.1 Ethical Approval and Sample Collection

Written, informed consent was provided by all human donors involved in this study. Uninfected blood donations were obtained by a trained phlebotomist at the Boston Children’s Hospital. The Institutional Review Board of the Institute of Biomedical Sciences at the University of São Paulo, Brazil approved the protocols for parasite sample collection (1169/CEPSH, 2014). *P. vivax* samples were collected in Mânio Lima, Acre State, Brazil, from *P. vivax* patients diagnosed via conventional thick-smear microscopy performed in the context of a randomized, open-label clinical trial [21], and the samples were depleted of leukocytes and cryopreserved as previously described [22].

4.3.2 Reticulocyte Purification

One unit of whole blood (~ 400 mL) from a hemochromatosis patient donor was obtained and leukocytes were removed using a Sepacell® R-500(II) filter (Fenwal 4C2300). Reticulocytes were enriched from the sample using a two-step procedure: first the whole blood in serum was spun at 4000 *g* for 2 hours and the top 25% of cells were saved. This process was repeated once, and then samples were resuspended at 50%
hematocrit in autologous serum. Aliquots of 6 mL of this suspension were layered on 6 mL of 1.080g/mL KCl Percoll in 15 mL conical tubes prepared as previously described [5]. These were centrifuged at room temperature at 1200 g for 15 minutes with slow acceleration and brake as previously described. The interface bands of cells from each tube was isolated and pooled. All subsequent procedures were performed at 4°C or on ice. Cells were washed 3x in PBS + 10% FBS + 2IU/mL heparin and counted using a MACSQuant (Miltenyi) flow cytometer. Cells were diluted to 2.0 x 10^7/mL in PBS + 10% FBS + 2IU/mL heparin and then labeled with CD71-APC (Miltenyi) for at least 10 minutes at 4°C covered from light. Cells were washed once and then resuspended in PBS + 10% FBS + 2 IU/mL heparin + 1 μg/mL thiazole orange. Cells were sorted on a SORP ARIA (BD) into five fractions based on different levels of CD71 and RNA staining.

4.3.3 Labeling reticulocyte surface proteins

Labeling of cells with TMT reagents and subsequent bioinformatics analysis was performed as described previously [23-26]. Briefly, two sets of 2 x 10^7 cells from each of the five sorted reticulocyte factions were labeled with aminooxy biotin which reacts with sialic acid residues on the cell surface [27]. Following quenching of the reaction, acetylation with iodoacetamide and triton X-100 cell lysis, the biotinylated proteins were isolated on streptavidin microbeads and subjected to extensive washing to reduce contamination. On-bead trypsin digestion was then performed, and the tryptic peptide fragments were labeled with a 10-plex TMT labeling kit (Invitrogen) with one TMT reagent per sample. After the labeling reaction, the 10 reticulocyte digest pools (duplicates of P1-P5) were pooled in a 1:1:1:1:1:1:1:1:1:1 ratio. Peptides fractions were separated on an
HPLC and analyzed using a MultiNotch MS3-based approach [23, 27]. Mass spectra data were processed, and peptide matches against the Uniprot human proteome database was performed [23].

4.3.4 Bioinformatics Analysis

The initial set of 489 identified proteins were analyzed as follows. The data were analyzed via $k$-means clustering using an Euclidian distance metric using Cluster v. 3.0 [28]. To determine the optimal number of clusters to use, we implemented a modified SSE method where the sum of the squared difference was taken between the sample value and the average of the technical replicates, and we observed a decrease in modified SSE value until $n = 6$ clusters. Proteins in the sixth cluster showed an unusual patter with high S/N coming mostly from one technical replicate, suggesting possible contamination. We excluded these proteins from further analysis bringing down the number of samples down to 407. Next we renormalized the S:N data, matching the sums of the S:N signal for each lane and re-clustered with $n = 5$ clusters using $k$-means.

In order to identify membrane proteins from this set, we used a combination of different analyses. First we searched the UNIPROT database [30] entries and we identified 262 proteins with a Gene Ontology (GO) “membrane” term. In parallel we analyzed the COMPARTMENTS [31] database entries for each protein and identified 166 proteins with a membrane score of 3 or more out of 5. From this analysis we found 158 proteins in common between the GO and COMPARTMENTS data. For these 158 proteins, we obtained the full-length protein sequences from the UNIPROT database [30] and used these sequences to predict transmembrane helices using two prediction
algorithms: TMHMM 2.0 [32, 33] and TOPCONS [34]. We also searched the UNIPROT database for curated membrane protein classification terms including: type I, type II, type III, type IV, multipass, GPI-anchored and lipid anchored. We identified 82 proteins that matched at least one of these criteria.

4.3.5 *P. vivax* ex vivo Culture and Marker Detection

Leukofiltered blood isolates from separate Brazilian patients were thawed and enriched on a 1.080 g/mL KCl Percoll gradient as previously described [11], and the enriched parasites were plated at 1 x 10⁸ cells per mL (~1% hematocrit) in indicated culture media supplemented with 10% v/v AB⁺ heat-inactivated human serum pooled from 15 human donors and 50 µg/mL gentamicin. Parasites were static cultured at 37°C in mixed gas of 1% oxygen, 5% carbon dioxide, balance nitrogen. At indicated times, approximately 1 x 10⁷ cells were collected for antibody staining with the following antibodies and concentrations for one hour each at room temperature in the dark: CD71-APC, Miltenyi, 1:100; CD36-VioBlue, Miltenyi, 1:100; CD49d-PE-Vio770, Miltenyi, 1:100; CD98-PE, Miltenyi, 1:100; CAT1-Rabbit, Sigma, 1:25; CLCN3-Mouse, Abcam, 1:25; KIAA1467-Rabbit, Abcam, 1:25; SLC12A6-Rabbit, Abcam, 1:25; anti-Rabbit-AlexaFluor405 secondary, ThermoFisher, 1:25; anti-Mouse-AlexaFluor405 secondary, ThermoFisher, 1:25; Vybrant DyeCycle Green, ThermoFisher, 1:10000. Samples were read on a MACSQuant (Miltenyi).
4.4 Results

To identify novel surface protein markers of various reticulocyte populations, we undertook an unbiased, surface proteomics approach. Circulating cells from a hemochromatosis donor were stained for nucleic acid content and CD71 and various subpopulations (P1-P5) were isolated using FACS (Figure 4.1A). The surface proteins of these subpopulations were labeled and isolated then identified and quantified by tandem-mass-tag (TMT) mass spectrometry. 407 proteins were detected in total and 158 were confirmed to have either Gene Ontology (GO) membrane classification or COMPARTMENTS plasma membrane classification (Figure 4.1B), and these were further characterized by having either transmembrane helices or lipid anchors (Figure 4.1C). The 158 were organized into 5 different k-means clusters (Figure 4.1D), with the top two clusters representing proteins that are rapidly lost through early reticulocyte maturation (Figure 4.1E). Of the proteins in these two clusters, a subset was chosen based on rate of loss through maturation, presence of transmembrane or lipid anchor predictions and reagent (antibody) availability (Table 4.1).
Figure 4.1 | Quantitative surface proteomics identifies proteins that change in abundance during reticulocyte maturation. A) Flow cytometry plot of peripheral blood reticulocytes stained with the RNA-specific dye thiazole orange and CD71 showing the five different populations (P1 - P5) that were purified by FACS. B) Classification of 407 identified proteins based on gene ontology (GO) [16-18] membrane classification (cyan) and COMPARTMENTS [19] plasma membrane classification (yellow). C) The 158 plasma membrane proteins identified in (B) were classified based on the presence of transmembrane helices, either as determined computationally via TMHMM2 [20] (blue) or via annotation from the UNIPROT database [18] (green). In addition, lipid anchored proteins were identified based on UNIPROT annotations (orange). D) The 158 membrane proteins were organized into five different clusters using k-means clustering [1] using normalized data. Columns are arranged as technical replicates of P1 to P5 (annotated based on (A)). Clusters 1 and 2 show decrease in relative protein abundance from P1 to P5. Clusters 3 and 4 show either no change or modest change in relative protein abundance from P1 to P5. Cluster 5 shows increased relative abundance in P5 relative to other fractions. E) Data from clusters 1 and 2 are shown with the log2-normalized fold change (calculated by comparing the ratio of signal:noise to the average S:N of the P5 technical replicates). Color-coded membrane protein annotations from (C) are also included. Asterisks represent proteins included in follow-up studies.
Table 4.1 | Selected Surface Markers of Reticulocyte Subpopulations

<table>
<thead>
<tr>
<th>TFRC (CD71)</th>
<th>SLC3A2/SLC7A5 (CD98)</th>
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</thead>
<tbody>
<tr>
<td>SLC12A6</td>
<td>SLC7A1 (CAT1)</td>
</tr>
<tr>
<td>CD36</td>
<td>CLCN3</td>
</tr>
<tr>
<td>CD49d</td>
<td>(FAM234B) KIAA1467</td>
</tr>
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</table>

To characterize the relationship between *P. vivax* and the subpopulations of reticulocytes defined by these reticulocyte markers we first quantified each subpopulation in *P. vivax*-enriched blood from Brazilian patient donors. This analysis demonstrated that there was a wide range of percentages of host cells that were positive for each marker, ranging from CD71⁺ cells averaging about 1.5% of the population to CD49d⁺ cells, which represented less than 0.05% (Figure 4.2A). Next, using DNA dyes we specifically label parasite-infected cells from enriched Brazilian donations and quantify both parasitemia and the marker-positive cells using flow cytometry. We measure the selectivity of the parasite for the various subpopulations of host cells, with selectivity representing the fold increase in marker and parasite double-positive cells over what would be expected assuming invasion is completely stochastic. Strikingly, SLC12A6, a potassium chloride (KCl) cotransporter also known as KCC3, shows significantly higher selectivity at approximately 64, compared to CD71 at approximately 20 (Figure 4.2B). Furthermore, to ensure the enrichment was not an artifact of using Percoll density gradients high in KCl for parasite enrichment, we performed the same analysis using unenriched Brazilian donations, and the selectivity of *P. vivax* for SLC12A6⁺ cells remained significantly higher compared to CD71⁺ cells (Figure 4.2C).
Figure 4.2 | Characterization of selected reticulocyte markers on cells from Brazilian clinical isolates.}

A) The percentage of cells positive for each marker in KCL Percoll-enriched samples recovered from cryopreservation. Error bars represent s.e.m. and p values are based on paired Student's t-tests between CD71 and each other marker.

B and C) the selectivity, defined as the fold-increase in percentage of cells positive for both P. vivax infection and each marker over the expected double-positive percentage assuming completely random invasion for both P. vivax infection and each marker and percentage of cells positive for each marker in KCL Percoll-enriched samples recovered from cryopreservation. B and C) the selectivity, defined as the fold-increase in percentage of cells positive for both P. vivax infection and each marker and percentage of cells positive for each marker in KCL Percoll-enriched samples recovered from cryopreservation. A) The
Next, to test for differences in the survival and maturation of *P. vivax* within SLC12A6<sup>+</sup> cells and CD71<sup>+</sup> cells, parasites in each cell population were monitored over the course of 44 hours of the IDC. Of the parasitized host cells at each time point, those positive for SLC12A6 begin as a larger fraction compared to those positive for CD71, and they quickly become the dominating proportion over time (Figure 4.3A). This is in contrast with the rapid loss of SLC12A6<sup>+</sup> cells as a proportion of the uninfected population (Figure 4.3B). In order to determine if the increase in the fraction of parasites in SLC12A6<sup>+</sup> cells over time is due to a gain of SLC12A6<sup>+</sup> cells a loss of other cell types, the *P. vivax* in various host cell subpopulations at each point of maturation was examined relative to the *P. vivax* at the initial time point. As seen previously, the majority of initially present *P. vivax* parasites tend to disappear over the course of 44 hours of *in vitro* maturation (Figure 4.3C) [5, 20]. However, this loss appears to be mostly accounted for by *P. vivax* in host cells negative for both SLC12A6 and CD71, while the majority of parasites within SLC12A6<sup>+</sup> cells appear to survive up until the latest stages through 44 hours of maturation (Figure 4.3C). Again, this is contrasted in the parasite negative population where the SLC12A6<sup>+</sup> and CD71<sup>+</sup> cells appear to convert, or mature, to SLC12A6⁻/CD71⁻ normocytes without any noticeable loss in cell total over time (Figure 4.3D).
Figure 4.3 | \( P. \) vivax within SLC12A6+ host cells are more likely to survive, and this depends on SLC12A6 function.

A and B) Percentage of infected cells (A) or uninfected cells (B) with each marker combination at each time point over the course of 44 hours of ex vivo maturation of KCl Percoll-enriched Brazilian isolates. Error bars represent s.e.m. of five biological replicates. C and D) Ratios of infected cells (D) and uninfected cells (E) with each marker combination, normalized to the initial time point. Error bars represent s.e.m. of total infected or uninfected cell numbers relative to the initial time point of five biological replicates. C) Parasitemia and marker combination ratios relative to the initial time point of parasites grown with and without 100µM KCC inhibitor, DIAO. Error bars represent s.e.m. of five biological replicates. D) Comparison of each marker subpopulation before and after maturation with and without 100µM DIAO. Error bars represent s.e.m. of five biological replicates. D) Comparison of each marker subpopulation before and after maturation with and without 100µM KCC inhibitor, DIAO. Error bars represent s.e.m. of total infected cell numbers relative to the initial time point of parasites grown with and without 100µM KCC inhibitor, DIAO. Error bars represent s.e.m. of five biological replicates. E) Parasite-positive RBCs at each time point with each marker combination. Error bars represent s.e.m. of total infected cell numbers relative to the initial time point of five biological replicates. F) Parasite-negative RBCs at each time point with each marker combination. Error bars represent s.e.m. of total infected cell numbers relative to the initial time point of five biological replicates.

\( p = 0.0307 \)

* \( p < 0.05 \)

**** \( p < 0.0001 \) by two-way ANOVA. Error bars represent s.e.m. of five biological replicates. Error bars represent s.e.m. of five biological replicates. Error bars represent s.e.m. of five biological replicates. Error bars represent s.e.m. of five biological replicates. Error bars represent s.e.m. of total infected cell numbers relative to the initial time point of five biological replicates.
Finally, to test if the functionality of KCl cotransporters is necessary for the observed enhanced survival of *P. vivax* within the SLC12A6+ cells, parasites were matured with and without the KCC inhibitor, DIAO, and the survival of *P. vivax* within various host cell subpopulations was evaluated after 44 hours *ex vivo* maturation. This reveals that there is a statistically significantly increased loss of *P. vivax* when matured in the presence of DIAO (Figure 4.3E). To understand if this overall decrease in survival was accounted for by the loss of *P. vivax* in specific subpopulations of reticulocytes, we examined the difference in survival between DIAO-treated and untreated parasites of each subpopulation. Over maturation, there was a large decrease in *P. vivax* within SLC12A6-/CD71- cells, but no significant difference in the endpoint survival of *P. vivax* within this population with and without DIAO treatment (Figure 4.3F). However, there is a difference in survival of the parasites within SLC12A6+ host cells, with a significant decrease in survival with DIAO treatment (Figure 4.3F).

Because these finding implicate the maintenance of ion concentration across host cell plasma membrane as important for *P. vivax* survival, the concentrations of major ionic constituents of various commercially available defined culture media were compared including the *P. vivax*-supportive medium, IMDM, the culture media traditionally used for *P. vivax* and other *Plasmodium spp.*, McCoy’s 5A and RPMI 1640, as well as a culture media formulaically similar to IMDM, DMEM (Table 4.2). From the comparison, it is clear that IMDM has the lowest concentration of the monovalent ions chloride and sodium, which make up the majority of the ion species. Of the ions that make up a relatively smaller concentration, IMDM has also a relatively slightly reduced concentration of potassium ions but is elevated in the divalent cations of calcium and magnesium.
Finally, to test if the media components that are increased in DMEM or reduced/absent in IMDM are important for *P. vivax* survival, the media components of each media was supplemented individually to DMEM or IMDM to reach the concentrations present in IMDM or DMEM respectively (Figure 4.4). It is clear that the addition of no individual component of IMDM to DMEM significantly enhanced the survival of *P. vivax* in the DMEM media. Conversely, only the addition to IMDM of sodium chloride to the levels present in DMEM greatly decreased the parasite survival through the IDC, further implicating ion concentrations as an important factor in *P. vivax* *ex vivo* culture.
Figure 4.4 | Reciprocal supplementation of DMEM and IMDM with individual ingredients of each culture media. Each indicated ingredient was individually added to DMEM to reach IMDM concentrations (Left) or individually added to IMDM to reach DMEM concentrations (Right). Enriched Brazilian *P. vivax* isolates were cultured in the resulting media and growth was measured via the tritiated-hypoxanthine assay. These data represent a single biological replicate, and error bars on the control conditions represent SEM of technical duplicates.
4.5 Discussion

This study identified various proteins that are selectively present on subpopulations of reticulocytes at various stages of maturation, especially the earliest stages. One of these proteins, SLC12A6 a potassium chloride cotransporter (KCC), is present on *P. vivax*-infected cells from Brazilian patient donors at much higher frequency than if *P. vivax* invasion into this subpopulation were stochastic. Moreover, *P. vivax* parasites within SLC12A6+ host cells tend to survive *ex vivo* maturation over 44 hours better than parasites in SLC12A6- cells, and this enhanced survival is abrogated specifically within this cell population when the culture is treated with DIAO, an inhibitor of host KCl cotransporters. Finally, we show that *P. vivax*-supporting IMDM culture has a reduced concentration of major ion species, including sodium, chloride and potassium, compared to less supportive media, and increasing the sodium chloride levels in IMDM decreases *P. vivax* survival.

The proteomic profiling of reticulocytes versus mature erythrocytes has been the subject of numerous other studies, with some even looking at protein changes in erythroid cells over the course of *in vitro* maturation from hematopoietic stem cells to reticulocytes [14, 35-38]. Although *in vitro* matured reticulocytes may indeed be a solution to sourcing cells for *P. vivax* culture in the future, making the previous characterizations of these cell types important [39], understanding the heterogeneity of the natural reticulocytes present in circulation may help identify host cell characteristics necessary for successful *P. vivax* culture, thereby informing proper cultivation of *in vitro* reticulocytes. This present work is the first study to characterize the surface proteomes of reticulocyte subpopulations of various ages that are contemporaneously extant in the circulating blood of a patient.
donor, presumably the subpopulations of host cells naturally available for *P. vivax* infection. Indeed, although only one reticulocyte donor was used, this study was successful in identifying many surface proteins that rapidly decrease through increasingly mature reticulocyte subpopulations. While only a small subset was selected for continued investigation, largely due to reagent availability, there may be additional surface proteins that were not pursued that could be functional in supporting *P. vivax* or nonfunctionally delineate an even more suitable host cell subpopulation.

The finding that host cells expressing SLC12A6 better support *P. vivax* intraerythrocytic maturation than other cells is interesting, and the possibility that SLC12A6 might not only delineate a *P. vivax*-supportive subpopulation of host cells but may actually be functionally important in the health of *P. vivax*-infected cells is compelling. Human KCCs were initially discovered in 1985 in the erythrocytic membrane, and they have since been implicated as a predominant factor contributing to erythrocyte cell-volume homeostasis in response to environmental osmotic changes [40, 41]. Of the four KCC-family proteins expressed during the human erythropoiesis, KCC3, encoded by SLC12A6, appears to be the predominant one both transcriptional and protein level [42]. The intracellular osmolarity and volume regulation via SLC12A6 is particularly interesting in the context of *P. vivax* intraerythrocytic infection. It is well documented that even with the use of various improved media for culture, there remains a significant death of parasites as they mature ([Figure 2.5]) [5, 20]. Furthermore, instead of becoming pyknotic and lingering within the RBCs as is the phenotype of many cases of *Plasmodium* death, the dead *P. vivax* parasites themselves are completely disappearing. This suggests that either the parasites are being selectively removed from the iRBCs, which is unlikely
especially without co-culturing the parasites with macrophages, or the more-likely case is that the iRBCs are being entirely destroyed, which osmotic stress could certainly achieve.

The osmotic stresses of intraerythrocytic *Plasmodium* development are well-documented, and in the case of *P. falciparum*, hemoglobin digestion is suggested to be a required process largely because it reduces the impermeant solute concentration within the iRBC, effectively equalizing the osmotic potential across the RBC membrane [43]. Interestingly, *P. vivax* may not have the ability to process hemoglobin as effectively as *P. falciparum*, as evidenced by the relatively smaller pools of hemozoin visibly present *P. vivax* via microscopy. Though, one group has quantified *P. vivax* hemozoin production as similar to *P. falciparum*, the quantification of hemozoin between the two species was not performed in the same experiments [44]. Additionally, it has been shown that even *P. falciparum* can replicate within reticulocytes without hemozoin formation, suggesting the reticulocyte provides a more osmotically favorable environment for *Plasmodium* growth in the absence of hemoglobin digestion [45].

Therefore, it is possible that to overcome a potential reduced efficiency in hemoglobin digestion, *P. vivax* reticulocyte tropism may be integral in the osmotic stability and therefore the survival of the parasite and associated host cell. Indeed, the reticulocyte may be osmotically protective for many reasons including the increased presence of SLC12A6 on the surface of reticulocytes, which may permit more reactionary potential of the infected cell to intracellular osmotic stresses brought on by the developing parasite (Figure 4.3) [42]. Also, because a significant portion (~20%) of hemoglobin has yet to be translated within the reticulocyte, these cells have a lower internal impermeable solute concentration that may provide more osmotic space for *P. vivax* development [46].
Additionally, it is important to weigh the fact that IMDM is formulated with generally lower monovalent ion concentrations than the less *P. vivax* supportive media combined with the fact that adding sodium chloride to IMDM to reach DMEM concentrations is the only individual media component to have a significant effect on *P. vivax* survival. This further associates the ionic status of the *in vitro* culture environment as extremely critical to parasite survival and also potentially relates to hemoglobin digestion. Although not particularly well studied with immature reticulocytes, in uninfected mature erythrocytes, hemoglobin acts an impermeant anion that displaces more permeant ions, namely chloride, from the host cell cytosol [47]. Therefore, a lower intracellular hemoglobin concentration in reticulocytes may increase the relative intracellular chloride concentration. This larger chloride pool may enhance the ability of the host cell to respond to osmotic stress by expulsing more chloride and potassium ions from the intracellular environment through the SLC12A6 protein. If the extracellular concentration of chloride or potassium is too high, as may be the case with the poorer culture media, the media may impede to the ability of SLC12A6 to push these ions from the cell. However, additional studies linking the media concentration of ions to the functionality of SLC12A6 in *P. vivax* survival will be necessary to fully understand the role these ions have in host cell stability.

Overall, the identification of proteins specific to young circulating reticulocytes may help contrast the biologic differences between reticulocytes and mature erythrocytes and has been useful in classifying novel host cell subpopulations among the heterogeneous reticulocytes. While we show *P. vivax* parasites recovered from cryopreservation are selectively present in SLC12A6+ host cells, further studies are needed to understand
whether this is due to enhanced stability of SLC12A6\(^{+}\) cells through cryopreservation and thawing, or if \(P.\ vivax\) parasites selectively invade SLC12A6\(^{+}\) cells inherently. Furthermore, additional studies are needed to determine if providing purified SLC12A6\(^{+}\) cells to \(P.\ vivax\ in vitro\) can boost invasion and subsequent survival and bring the field closer to continuous and robust \(P.\ vivax\ in vitro\) culture.
4.6 Acknowledgements

We would like to sincerely thank the patient donors, donor families and sample collection staff for providing the invaluable parasite resources necessary for this study. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication. The authors declare that they have no competing interests.
4.7 References


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CHAPTER FIVE

Discussion
5.1 The importance of understanding the features of culture media that support and/or inhibit \( P. \text{ vivax} \) ex vivo survival

*In vitro* culture for *Plasmodium falciparum*, the deadliest species of human malaria, has been central to the marked reduction in *P. falciparum* cases witnessed over the past couple of decades, and it will certainly be central in the coming decades as a shift in focus toward global malaria eradication calls upon new large-scale compound screens, vaccine development and drug target discovery to achieve this goal [1-4]. However, the call for eradication does not put only *P. falciparum* in the crosshairs, but all of the *Plasmodium* parasites that cause human malaria, including *P. vivax*, which is the most geographically widely distributed human malaria parasite, and which causes the majority of malaria cases outside of Sub-Saharan Africa [5]. However, the field of *P. vivax* research is set back by the lack of a continuous and robust *in vitro* culture system for the blood stages of this parasite. Although we have clearly demonstrated in Chapter Two that Iscove’s Modified Dulbecco’s Medium (IMDM) approximately doubles the survival of *P. vivax* through the initial round of *ex vivo* maturation, despite many attempts to understand the critical components of IMDM that provide this boost in survival, we still have no definitive answer.

While culture media certainly provides essential nutrients to whatever organism is being cultivated, other characteristics of the media play crucial roles in establishing a growth-enabling environment, including the media acidity and buffering methods/capacity as well as the ion concentration and resulting osmotic and electric potentials, and each of these have been shown to be important for *Plasmodium spp.* cultivation [6-8]. The finding that IMDM robustly enhances the survival of blood stage *P. vivax* parasites
provides a positive experimental control that never existed before, a control that will help in the design of experiments focused on elucidating the extracellular environmental requirements of *P. vivax*. To simplify a complicated problem, IMDM may be better for *P. vivax* for one or both of two reasons. First, it may provide something additional that the parasite requires for survival that other media do not provide. This is easily tested by adding in components of IMDM to various culture media and measuring the resulting parasite survival. This was done in various iterations (Figure 2.4), but this approach had little success in identifying the critical components of IMDM, which may be because there are many components of IMDM working in combination that are necessary for the boost in *P. vivax* survival. Alternatively, the second reason IMDM may be better is that it may have less of a particular component or components that are present in other media that may be detrimental to *P. vivax* survival. This is more difficult to directly assess as the ideal experiments would involve testing survival in culture media with various components removed, which requires precise formulation of media by the experimentalist or the purchase of commercially validated formulations, which is highly expensive.

Nevertheless, putting more effort toward elucidating media characteristics key for *P. vivax* survival is undoubtedly warranted for multiple reasons. One reason is to gain an understanding of the biological requirements of the parasite so that they may be exploited for treatments. Perhaps the culture media requirements will highlight components and therefore metabolic pathways that are necessary for growth, and these pathways may eventually serve as antimalarial drug targets in the future. On the other hand, if it is found that certain components of the poorly performing media are detrimental to *P. vivax*
growth, these or versions of these components themselves could potentially serve as antimalarial interventions.

Another reason to elucidate the culture media characteristics key for *P. vivax* survival is that it will be remarkably important for informing the creation or choosing of even better, more supportive culture media. While the application of IMDM has boosted *P. vivax* survival, there remains a remarkable decrease in parasitemia over the first round of the *ex vivo* intraerythrocytic development cycle (IDC), indicating there may room for further improvement for the culture system, including the media. While supplementing IMDM with various additives in an attempt to boost survival was attempted unsuccessfully in this dissertation work (Figure 2.6), this was performed without any knowledge of key IMDM characteristics and was by no means an exhaustive set of additives. Applying the rapid and sensitive isotopic metabolic labelling assay, as described in Chapter Two, to assay *P. vivax* survival will greatly increase the throughput of future additive screening.

5.2 Clarifying the role of ion concentrations for *P. vivax* survival

Although this dissertation provides no definitive conclusions regarding the important characteristics of IMDM that are key in supporting *P. vivax* survival, there is much evidence that implicates the concentration of ions as highly influential. Indeed, it is remarkable that IMDM is the only commercial media we tested that significantly boosted parasite survival (Figure 2.2), and the only tested component of the IMDM formulation that was altered with effect on survival was sodium chloride concentration (Figure 4.4). This combined with the implication of SLC12A6, a potassium chloride cotransporter responsive to osmotic stress, as a functional marker predicting *P. vivax* survival clearly
implicates the importance of ion concentrations in this system (Figure 4.3). However, the specific roles of different ions, the role of hemoglobin digestion and the role of host and parasite transporters remains unclear.

Although adding sodium chloride to IMDM inhibits *P. vivax* growth, it is unclear how, but two possible explanations are that the sodium chloride inhibits the ability of the cells to respond to osmotic stress, or the sodium chloride changes the initial state of the cells to be more osmotically fragile, or a combination of these options. Perhaps the easiest explanation is that higher concentrations of ions found in less supportive *P. vivax* culture media may decrease the ability of SLC12A6 to respond to osmotic stress by increasing the gradient against which SLC12A6 would be pumping ions. This could theoretically limit both the rate and extent of osmotic response. A more complicated explanation is that higher sodium chloride concentrations may increase the initial volume of cells, reducing the tolerable range of osmotic stress. Naturally, chloride concentrations, coupled with bicarbonate concentrations, tend to be at electrochemical equilibrium between the intra and extracellular environment [7]. This suggests that increasing extracellular chloride concentrations may also increase intracellular concentrations, and thereby cell volume, and may even acidify the intracellular environment, which may make the cell unsuitable to host *P. vivax* parasites. Future *P. vivax* survival studies utilizing various ion species as stressors, accurately measuring cell volume responses, and carefully characterizing the mode of parasite death will be key in understanding the true role of ions and osmotic stability in *P. vivax* survival.

Furthermore, one of the most definitive conclusions we have from these studies is that the SLC12A6+ host cells are better at supporting *P. vivax* survival than SLC12A6−.
cells, and inhibiting potassium chloride cotransport abrogates the survival boost. Nonetheless, it is still uncertain why the ability of the host cell to respond to osmotic stress is so crucial for \textit{P. vivax} when it seems to be less crucial for non-reticulocyte-tropic parasites like \textit{P. falciparum}. One model is that while it is well known that \textit{P. falciparum} can alleviate some osmotic stress within the host cell by digesting hemoglobin, perhaps \textit{P. vivax} doesn’t have such a robust ability, and that is the reason it relies more heavily on host osmotic responses [9]. An alternative, but not mutually exclusive, model is that the reticulocyte host cell must support both the residual production of hemoglobin as well as the intraerythrocytic maturation of the parasite simultaneously, and these processes together present a rapidly changing osmotic environment within an infected reticulocyte that requires combined response of hemoglobin digestion and SLC12A6 action to cope [9, 10]. Future studies utilizing more KCC inhibitors, genetic overexpression and knockout/down of SLC12A6, and modulating the ability of hemoglobin production by the reticulocyte and digestion by the parasite will be necessary and may even provide insight into why \textit{P. vivax} has such a restrictive tropism for reticulocytes.

5.3 Capitalizing on the current advancements

5.3.1 Fully exploiting the power of cryopreserved malaria isolates

Although this study does not report success in the ultimate goal of establishing continuous and robust \textit{P. vivax} \textit{in vitro} culture, it does provide a strong technical foundation upon which many studies can build by utilizing the enhance \textit{ex vivo} survival of cryopreserved isolates and refined maturation assays. Indeed, this dissertation work has enabled the robust enrichment of \textit{P. vivax} from relatively small volume cryopreserved
isolates for subsequent experimentation with accurate quantification. As mentioned previously, the use of fresh primary isolates is extremely resource-intensive, requiring both a clinical and research laboratory infrastructure that support sample acquisition and manipulation, all in *P. vivax*-endemic regions with are nearly universally resource-poor settings [35]. However, by enhancing the utility of cryopreserved isolates, we take away the stipulation of a fully equipped lab nearby to the clinic, since leukocyte filtering, sample aliquoting and freezing would be the only functions necessary from the nearby laboratory. This allows research groups anywhere in the world to begin investigating *P. vivax* biology as long as they have a source of cryopreserved isolates that can be shipped to their labs. An obvious way to obtain isolates would be to collaborate with groups who are already collecting them for ongoing studies, which is how we obtained many of our isolates. However, I propose that establishing centers of sample collection for international research through various malaria endemic regions is a more effective option.

If a unifying organization, such as the United States NIH or the WHO, established sites dedicated, at least in part, to the collection of human malaria samples over time for the explicit purpose of distributing those samples to research groups, many hurdles to malaria research could be alleviated. First, these organizations could work with existing clinical locations, such as the ICMER sites, to supplement personnel and resources, minimizing the start-up costs of such centers and minimizing negative impacts on currently ongoing research. These centers for sample collection would also maximize efficiency, utility and confidence by specializing in recruiting patients in their respective locations, obtaining informed consent, gathering and curating patient, geographical and historical information, sample processing and cryopreservation, and the storage and
shipment of the cryopreserved isolates. The database surrounding the sample collection itself would be an invaluable resource for epidemiologic studies, but this would also provide an incredible resource of primary isolates that could be used for longitudinal studies of parasite evolution, cross sectional studies of drug sensitivity, comparison of parasites within and across geographic regions and many other pursuits. Furthermore, this should not be exclusive to just *P. vivax* where primary isolates are the only current method of studying the parasite; *P. falciparum* and other human malaria parasites would greatly benefit as well.

### 5.3.2 Maximizing the use of robust *P. vivax* in vitro maturation

By increasing the quantifiability and survival of *P. vivax* through even just one round of maturation so that schizonts can be robustly cultivated, this study has unlocked the ability to apply experimental techniques that can answer important questions now, even without a continuous and robust *in vitro* culture system. First, as reported in Chapter Two, we have adapted the isotopic metabolic labeling assay using tritiated hypoxanthine for the rapid and sensitive screening of *P. vivax* maturation, which is useful for antimalarial assays. In a time when resistance to chloroquine, the first line antimalarial against *P. vivax*, is documented in nearly every endemic region, this assay could help to not only monitor for the spread of chloroquine resistance, but also help to differentiate chloroquine sensitive and insensitive populations toward the goal of understanding the mechanism of chloroquine resistance, information that remains elusive for *P. vivax* [11, 12]. Furthermore, in the spirit of establishing continuous and robust *P. vivax* in vitro culture, this rapid maturation assay could be used to screen a wide variety of potential enhancers
of parasite survival. From particular nutrients to epigenetic modifiers, these screens could encompass a wide range of effectors that may boost *in vitro* survival and elucidate biology along the way.

Another assay the robust maturation of schizonts permits is the study of *P. vivax* invasion. While Chapter Four of this dissertation somewhat elucidates the niche of *P. vivax* among the heterogeneous reticulocyte population, further work is still needed to understand if the SLC12A6$^+$ cells or other subpopulations can serve as receptor cells for robust *in vitro* reinvasion and subsequent survival. Although it is likely a necessary approach, purifying cells with the various reticulocyte markers from circulating blood is an resource-intensive and laborious process as they represent a fraction of reticulocytes, which themselves represent a small fraction of circulating cells. An alternative approach will be to generate reticulocytes *in vitro* using primary CD34$^+$ hematopoietic stem cells or established immortalized erythroid cell lines, and our *P. vivax* maturation processes unlock the ability to test these for invasion capabilities [13, 14]. Finally, although many invasion ligand-receptor pairs have been identified for *P. falciparum*, only two, CD71-*PvRBP2b* and DARC-*PvDBP*, have been identified for *P. vivax* [15-17]. Combining genetically tractable erythroid systems with our robust schizont maturation, future studies are empowered to examine novel ligand-receptor interactions, information that will be important in both establishing continuous and robust culture and identifying potential vaccine targets.
5.4 Concluding remarks

In total, this goal of this dissertation was to move the field closer toward continuous and robust \textit{in vitro} culture of \textit{P. vivax} blood stages through developing a better understanding of various aspects of its intraerythrocytic biology. We successfully develop techniques to enrich, mature, and quantify the parasite that will, in themselves, be important in future studies advancing the biologic understanding of this parasite. Furthermore, we utilize these techniques to investigate the transcriptional profile of the parasite, establishing the first transcriptome from purified \textit{P. vivax} gametocytes which is important for identifying markers differentiating sexual from asexual stages and identifying potential transmission blocking vaccine candidates. Additionally, we discover surprisingly little parasite transcriptional response to the culture media, implying that more focus is needed on the importance of the health of the host cell. Finally, we identify a potassium chloride cotransporter on a subpopulation of reticulocytes that appears to be functional in supporting the successful maturation of the parasite, implicating ionic balance as crucial to \textit{P. vivax} survival. Together, this dissertation highlights numerous unique biologic characteristics of this parasite, empowers future courses of study to be more quantifiable, reproducible and rapid, and may be a significant stepping stone toward continuous and robust \textit{in vitro} culture of \textit{P. vivax}. 
5.5 References


