Development of a flow cytometry based assay to quantitatively investigate *P. falciparum* erythrocyte invasion

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August 2009

The dissertation is submitted for the degree of

Master of Philosophy in Biological Sciences

Preface

The work included here is my own and includes nothing which is the outcome of collaborative work, unless indicated otherwise within the text.

Acknowledgements

I would like to thank my supervisor Julian Rayner for his advice and keen interest in the progress of this project as well as my personal development. I would also like to acknowledge members of Team 115, in particular Michel Theron and Matthew Jones for their daily assistance and advice. In addition, I would like to thank members of Team 131 for sharing an office and in particular Katrin Volkmann for her assistance with microscopy. Other exceptionally helpful individuals include Bee Ling Ng and Nigel Carter for assistance and permission to use their flow cytometer, Christine Hale and Gordon Dougan for assistance and permission to use their flow cytometer, and finally Bronwyn MacInnis and Dominic Kwiatkwoski for their interest and contributions that enabled this research opportunity. I would also like to thank Oliver Billker and Matt Higgins for their time and presence on my thesis committee as well as Alex Bateman, Christina Hedberg-Delouka, and Annabel Smith for their support with degree related advising. Also I would like to acknowledge the outstanding group of Sanger PhD students with whom I was fortunate to join in their first year of training, most notably Samuel Ayalew Assefa for guidance and wisdom on all things computational. Finally, I would like to acknowledge the Gates Cambridge Trust for their support of my studies here at Cambridge, as well as Darwin College and the Wellcome Trust Sanger Institute.

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Abstract

Plasmodium falciparum invades human erythrocytes in a central step in the molecular pathogenesis of malaria, one that marks the beginning of the stage of the parasite life cycle associated with the clinical presentation of malaria. The molecules and the mechanisms utilized to accomplish the complex, multi-stage process of invasion are thought to be important in the context of development of novel anti-malarial therapeutics. It has long been known that parasites utilize various parasite ligand and host erythrocyte receptor pairs, termed invasion pathways, to gain entry into the erythrocyte. In both the laboratory and the field, parasites utilize a range of pathways that are differentially sensitive to various enzyme treatments of erythrocytes. To assess at large scale the significance of this variation as well as to enable basic investigation into the mechanics by which invasion pathway variation is generated, a novel flow cytometry based assay was developed. A two-step Hoechst 33324 flow cytometry protocol was developed to quantify parasitemia within fixed erythrocytes, yielding values consistent with the traditional Giemsa stained microscopy method and at significantly higher throughput. Reported here is also a multi-color combination method of PKH lipophilic erythrocyte labeling dyes with Hoechst 33324 staining of parasites for the purpose of specifically distinguishing invasion of parasites into the specified target cells for a given assay. PKH stains however appear to be measurably toxic with respect to invasion into unstained erythrocytes and require further optimization. However, with a rapid method to score parasite invasion using Hoechst and the use of traditional enzyme inactivation methods to prevent reinvasion into donor erythrocytes of an invasion assay, large scale invasion studies can now be performed and in the near future correlated to growing genome wide data sets and resources to infer previously unknown aspects of the biology of *P.falciparum* erythrocyte invasion.

Introduction

Plasmodium falciparum parasites cause the deadliest form of malaria causing approximately 500 million cases and over one million deaths in sub-Saharan Africa annually**.** Remarkable progress has been made recently with 29 countries worldwide reducing mortality caused by malaria by 50% in the past 8 years. However, as of 2007, it is estimated that 1.38 billion people remain at risk of contracting *P.falciparum* malaria in a stable transmission zone. Moreover there is no licensed anti-malarial vaccine and recent reports of declining efficacy of first-line artemisinin-based therapies, highlight the need for a better understanding of the mechanisms by which disease and immunity to malaria occurs (Dondorp et al., 2009; Hay et al., 2009). *Plasmodium falciparum* parasites belong to the phylum Apicomplexa, along with other disease-causing parasites such as *Toxoplasma, Cryptosporidium, Babesia* and *Theileria* spp. Within the Plasmodium genus itself it is estimated that there are thousands of species that show remarkable diversity in host specificity.

Despite this diversity, consistent across several apicomplexan parasites is the general localization of the machinery required for invasion, an essential process in the biology of these parasites. *P.falciparum* invades and develops in a series of insect and human cell types in a complex life cycle. Broadly the *P.falciparum* life cycle can be separated into the exoerythrocytic stage, initiated during the blood meal when mosquito salivary gland sporozoites are injected and go on to infect and develop in liver cells. They eventually rupture liver cells and invade erythrocytes, thereby beginning the erythrocytic asexual stage. Within the erythrocyte, the parasite grows and divides in a series of intraerythrocytic developmental stages (rings, trophozoites, schizonts) up till rupture of the erythrocyte and release of free merozoites. The cycle is completed within the human host by the transmission of gametocyte sexual stages back

to the mosquito during the blood meal. The sporogonic stage begins, as gametocytes become gametes and fuse to form a zygote that eventually becomes a motile ookinete that can migrate through the mosquito intestinal epithelia. They then become immobile oocysts that grow and eventually divide into sporozoites that migrate to the salivary gland to restart the life cycle with the next blood meal. Of all these stages, the intraerythrocytic stage is associated with the pathological symptoms of malaria. Understanding the biology of the process by which parasites invade into human erythrocytes and begin to establish themselves within the human erythrocyte environment, thereby causing disease, is important in the context of future development of antimalarial therapeutics including vaccines.

Invasion of *P.falciparum* into human erythrocytes involves four steps: attachment of merozoites to the erythrocyte, apical reorientation, tight-junction formation and subsequent entry into the erythrocyte forming a parasitophorous vacuole. This entire process involves a series of interactions, beginning with what is thought to be long-distance, reversible interactions of low affinity, followed by primary attachment at any given point along the erythrocyte. Once primary attachment has occurred, the merozoite reorients to bring the apical end of the merozoite closer to the erythrocyte surface (Bannister and Dluzewski, 1990). A tight junction is then formed between merozoite and erythrocyte surfaces which enables parasite entry as the junction migrates towards the posterior end of the merozoite (Keeley and Soldati, 2004). As this happens, certain proteolytic events are thought to occur that clear remnant surface ligands on the parasite-erythrocyte interface and additionally a parasitophorous vacuole is formed that separates the parasite from the host cytosol (Yeoh et al., 2007).

Several *P.falciparum* proteins are thought to play an important role in the various stages of invasion. In establishing initial contact with the erythrocyte up and till the stage of reorientation, well conserved proteins localized at the apical end of apicomplexan parasites Apical Membrane Antigen (AMA1) and to a less certain degree glycosylphosphatidylinositol (GPI) anchored merozoite surface proteins (PfMSP1) have been implicated (Cowman and Crabb, 2006; Triglia et al., 2000). At the time of tight junction formation, two families of parasite proteins are thought to be involved in initiating entry through parasite ligand and host receptor interactions. Members of the Duffy binding-like (DBL) protein family and *P.falciparum* reticulocyte binding protein homolog (PfRh) are thought to bind various host cell receptors, including glycophorin A, C and likely other host molecules yet to be identified. These proteins frequently show a high degree of polymorphism probably due to selection pressure from the immune system in routinely exposed populations.

These parasite ligand-host receptors are shown to be independent of each other, hence interactions are thought to constitute a range of pathways by which invasion can be directed. Despite the large number of proteins implicated in activating invasion after reorientation, many of the members of the DBL and RH families are non-essential and probably redundant (Cowman and Crabb, 2006). The versatility conferred by this redundancy is thought to allow the parasite to switch host receptor utilization conferring a selective advantage for invading diverse hosts, although the mechanisms for this hypothesis are yet to be fully elucidated (Stubbs et al., 2005).

Variation in the ability for parasites to invade diverse hosts has been modeled in vitro by studying the differential ability for parasites to invade erythrocytes treated by various enzyme treatments. Enzyme treatments deplete and vary the abundance and nature of the host erythrocyte

receptors made available to the parasite. An example of this modeled variation in vitro is the ability for some strains (e.g. 7G8) to invade neuraminidase-treated erythrocytes with little effect on invasion efficiency compared to wild type, but being unable to invade trypsin-treated erythrocytes (Perkins and Holt, 1988). These parasites are thought to utilize a sialic acid independent trypsin-sensitive receptor pathway. Conversely, the strain Dd2 is unable to invade neuraminidase treated erythrocytes but able to invade trypsin treated erythrocytes, using a sialic acid dependent, trypsin-resistant receptor to gain entry. Interestingly, under selection pressure by continual growth of initially poor growing Dd2 parasites in neuraminidase treated erythrocytes over several cycles, a stable switch to a sialic acid independent invasion pathway is elicited (Dolan et al., 1990). In a series of experiments using these enzyme treatments, various strains of parasites and glycophorin deficient erythrocytes, molecular participants of pathways began to be identified. More recent experiments, using gene disruption technology, demonstrate an in vitro shift in parasite utilization of neuraminidase-resistant and trypsin-resistant receptors towards neuraminidase sensitive and trypsin sensitive by the disruption of a single gene in the Rh family of proteins. These studies demonstrate how both variation in the parasite ligands and the availability of host receptors can be utilized to tease apart ligand-receptor interactions that direct the route by which invasion occurs (Duraisingh et al., 2003).

Interestingly, *P.falciparum* field isolates also exhibit a range of invasion phenotypes. A study of 15 field parasite isolates obtained from various parts of India showed that 12 of these used alternate invasion pathways that are neuraminidase resistant (Okoyeh et al., 1999). Invasion pathway variation in the field suggests that the presence of alternate/redundant pathways may be of clinical relevance. A study comparing parasites from 34 adult Thai patients with uncomplicated malaria compared with those from 42 patients suffering severe malaria,

demonstrated that initial cycle multiplication rates were 3-fold higher in the severe malaria group (Chotivanich et al., 2000). There is however little other evidence to correlate virulence of strains with less selective invasion phenotypes. In both of these previous studies, relatively few parasites were studied and it would be of interest to assess invasion pathways in a larger sample of parasites. This would further clarify the nature and in vivo significance of this phenotypic variation as well as potentially take into account other factors including endemicity and multiplicity of infection.

One of the principal reasons studies of invasion pathways in *P.falciparum* parasites have been limited in scale is the laborious process involved in performing invasion assays. Traditionally these assays are performed by incubating a preparation containing parasites, usually termed ‘donor cells’, with ‘target cells’, fresh uninfected erythrocytes which can often be enzyme treated. The donor erythrocytes are either subject to purification steps to isolate late stage parasites, schizonts, or alternatively donor erythrocytes containing early stage parasites are enzyme inactivated by a combination of trypsin and neuraminidase treatments. Both methods serve the same purpose of ensuring that the invasion assay only measures invasion of parasites originating from the donor erythrocyte population that subsequently invade into erythrocytes of the target population. These treatments are not trivial, particularly when working with a large number of individual invasion assays and treatment conditions of target cells. After incubating donor with target erythrocytes for usually 48 hours, erythrocytes from the invasion assay are thin smeared, Giemsa stained and infected erythrocytes scored manually using a light microscope to obtain a measure of invasion efficiency. This manual method has severe limitations on scale due to use of manpower and results could easily suffer from inconsistencies due to differences in user expertise and endurance levels.

Another limitation with traditional approaches is the relative difficulty by which invasion phenotypes can be correlated to specific differences at the functional molecular level. This limitation is perhaps in part self-imposed by the difficulties associated with applying the molecular biological toolkit to *P.falciparum* in vitro. To correlate invasion phenotypes with individual molecules typically involves validation by way of gene disruption, RT-PCR and potentially sequencing which makes it challenging to perform systematic unbiased analyses across the 23 MB and 5300 predicted genes in the *P.falciparum* nuclear genome (Gardner et al., 2002). To arrive at genotype-phenotype correlations, one would have to knock-out putative genes or create stable transgenic parasites to determine whether this affects utilization of the invasion pathway of interest. Furthermore, gene disruption or over expression imposes certain artificial constraints on the system that may not be representative of situations that these parasites and erythrocytes encounter in vivo. Another important consideration is that these conventional techniques typically disrupt or manipulate the physical interactions that occur at the surface of parasite and erythrocyte. Significant gaps that persist in our knowledge of invasion include the identities and mechanisms by which molecules that are downstream and upstream of the physically interacting participants of the invasion process that help to orchestrate invasion and invasion pathway switching.

An example of recent whole-genome systematic approaches to uncover the molecular basis of an interesting invasion phenotype is the use of a microarray approach to profile gene expression differences between mutant strains which reversibly switch between sialic acid dependent and sialic acid independent pathways. These studies reveal that activation of sialic acid independent invasion in the absence of the parasite protein EBA-175 is regulated by the activation of the PfRH4 gene (Stubbs et al., 2005). Further questions with regard to how PfRH4

gene expression is regulated remain and further unbiased, genome-wide approaches coupled to experiments that are designed based upon previous knowledge of redundant invasion pathways are likely to further illuminate the intricate mechanisms adopted by the parasite.

The Wellcome Trust Sanger Institute, since its early seminal role in generating 34% of the first whole genome *P.falciparum* genome sequence has recently embarked on the whole genome sequencing of parasite strains from around the world, as part of the Malaria Genomic Epidemiology Network (MalariaGEN) (Achidi et al., 2008). MalariaGEN consists of a consortium of malaria researchers from 21 different countries aimed at, among other objectives, developing an understanding of the genetic diversity of *P.falciparum* strains from around the world. Data emanating from these collaborative efforts are shedding light on the all important underlying parasite population structure and thereby providing a useful platform upon which genome wide association studies in *P.falciparum* can be performed. A high-throughput invasion assay, able to generate phenotypic data in parallel scale as current sequencing output, may provide a fruitful approach to resolve the molecular basis of invasion phenotype variation in an unbiased and statistically powerful approach.

The objective of the research project under discussion is to develop a novel invasion assay that addresses the two major limitations of scale and consistency by generating an accurate, large-scale assessment of invasion phenotypes of field parasite isolates. The first aspect of this project involves overcoming the limitations on scale imposed by user-call based microscopy, for which a flow cytometric method shall be developed. The second challenge is to specifically distinguish invasion events between parasites originating from donor cells into target cells incubated in a given invasion assay. Both aspects of the project aim to increase the

throughput, accuracy and convenience by which a large scale study of invasion pathways can be carried out.

The proposed flow cytometry approach takes advantage of the fact that erythrocytes are anuclear hence nucleic acid stains can be used to differentiate infected from uninfected erythrocytes. The resulting staining of invaded intracellular parasites can be scored in an automated fashion using flow cytometry. Flow cytometric based methods have already been employed using nucleic acid stains as well as transgenic green fluorescent protein (GFP) expressing *P.falciparum* parasites to profile activity of potential antimalarial compounds on parasite growth (Contreras et al., 2004; Sanchez et al., 2007). These methods appear to be replacing traditional approaches using optical microscopy and the use of radioactive nucleic acid precursors ^{3}[H]-hypoxanthine.

The second challenge in this project is to develop a cell labeling system to specifically distinguish invasion of parasites into the specified target cells of a given assay. Although a flow cytometry based protocol is likely to provide a scalable platform for scoring parasitized erythrocytes, on its own a simple DNA staining protocol would not be able to distinguish invasion into target cells from reinvasion into uninfected donor cells. By labeling the target cells and using multi-parameter flow cytometry to evaluate both the presence of erythrocyte label and DNA stain simultaneously, one could specifically distinguish invasion of parasites into target cells. This multi-parameter flow cytometry method also allows for further addition of layers of complexity as one may see fit, potentially using transgenic parasites and more specific labeling procedures, to achieve the goal of investigating the impact of natural and experimental genetic variation in both host and parasite on erythrocyte invasion at large scale.

Materials and Methods

P.falciparum culture

P.falciparum strains were cultured in O^+ human erythrocytes from donors at a range of hematocrits, either 2 or 5%, and 10% O^+ human sera as is conventionally performed (Trager and Jensen, 1976).

Flow Cytometry Based Quantification of *P.falciparum*

All live parasite work took place within the CL-3 contained tissue culture facility at the Wellcome Trust Sanger Institute. A bench-top Guava EasyCyte single 488nm laser instrument consisting of the following filters red, yellow, green was utilized for the development of DNA dye stain based method. For further assay development, work was switched to the BD LSR II instrument as part of the flow cytometry core facility located within a CL-1 space of WTSI. Several DNA dyes were tested, but of those that showed any promise after first testing and were completely tested include SYBR Green (Invitrogen) and Hoechst 33324 stains (Invitrogen). Maximum excitation/emission spectra are (497/520 nm) and (355, 465 nm) respectively. SYBR Green was excited by the 488 nm laser in both Guava cytometer and BD LSR II cytometer. Hoechst 33324 was excited by the 355 nm BD LSR II laser.

All parasite samples were analyzed by flow cytometry post-fixation due to health and safety precautions. Various fixatives were tested including glutaraldehyde, paraformaldehye and mixtures of both to demonstrate the effectiveness in rendering parasites inactive and safe to work with in CL-2 or CL-1 environment. Further procedures on fixed erythrocytes including permeabilization 10 min 1% Triton-X (Sigma) treatment, 1 hr RNase I (Sigma) treatment 1ug/ml

and incubation with various DNA stains were tested. Stained cultures were visualized using a fluorescent microscope (Leica DM 7500) to ensure that parasitized erythrocytes are being stained.

Optimized Hoechst protocol staining

A protocol without permeabilization and RNase treatment using a simple one-step staining procedure with the cell permeant Hoechst 33324 post-fixation was attempted, potentially cutting down processing time of samples by 1.5 hours. Various parameters including staining buffers (PBS and RPMI), staining temperature (25 and 37 ºC), concentration of dye (1, 2, 3 µM), and time of staining (5 min, 15 min, 1 hr) were optimized to obtain the best results defined by two criteria: degree of separation between infected and uninfected cells and correlation of flow cytometry staining protocol parasitemia with Giemsa stained microscopy parasitemia.

Erythrocyte Labeling

5% hematocrit blood is diluted in 1 ml of Diluent C mixed with an equal volume of Diluent C with a PKH dye dissolved in. The cell-dye suspension is rapidly and homogeneously mixed using a 1000 ml micropipette followed by an incubation period of 2 minutes (Sigma). The labeling reaction is stopped by the addition of an equal volume of 1% albumin dissolved in RPMI, followed by several wash steps using 10ml of complete media (including sera). Following the labeling reaction and subsequent wash steps, the cells can be resuspended in complete media for introduction into an invasion assay. PKH 26 and PKH 67 dyes were purchased from Sigma Aldrich. The maximum excitation/emission are (551/556 nm) and (490/502 nm) for each respectively and both were excited by the 488 nm laser on the BD LSR II.

Invasion Assays

Typically invasion assays were performed by incubating 50 µl of early stage 3D7 ring stage parasites (6 – 12 hrs post invasion) at 1 - 2% parasitemia, 2% hematocrit with an equal volume of uninfected, washed 2% hematocrit target erythrocytes for a period of 48 hours in CO_2 gas chamber at 37 ºC. Experiments were performed in 96 well plate format and typically performed in duplicate or in triplicate. After 48 hours, a portion of cells from each well was smeared, Giemsa-stained and ring stage parasites counted while another portion was fixed and processed for flow cytometric analysis.

Results

SYBR Green and Hoechst DNA dyes distinguishably stain parasitized erythrocytes using various protocols

SYBR Green and Hoechst 33342 (HO) DNA stains were shown to stain infected erythrocytes contained in parasite cultures on the BD LSR II. DNA dye stained samples run through the flow-cytometer distinguish into two distinct populations of cells on either the FITC or Indo-1-violet filters, as expected based on the emission spectra for SYBR Green and HO respectively (Fig. 1 & Fig. 2). The major population of cells on the left most side represents uninfected erythrocytes. The relatively smaller subset of events to the right of the background population represents the infected erythrocyte population which has retained the DNA dye. This population of cells, referred to as the shifted or excited population, is shifted due to excitation of the fluorochrome stain by one of the BD LSR II's lasers.

All four varied erythrocyte staining protocols tested including a 5 minute DNA-dye stain, 1 hour DNA-dye stain, brief permeabilization followed by 1 hour DNA-dye staining, and brief permeabilization, 1 hour RNase treatment followed by 1 hour DNA-dye staining with both dyes yielded two distinct populations of infected and uninfected, at varying degrees of clarity and separation. SYBR Green stained, uninfected erythrocyte control samples exhibit a degree of non-specific binding, referring to the haze of events associated with the background uninfected population of cells, with the exception for the RNase treated, SYBR Green stained sample. This haze is also found in the corresponding infected, stained erythrocyte control samples. In comparison, the uninfected, HO stained erythrocyte control samples do not show any haze. However, infected, HO stained erythrocytes that have been permeabilized do show a small haze

of events between the infected and uninfected populations, which disappears in a protocol that adds on RNase treatment.

In terms of separation between infected and uninfected erythrocytes, across all protocols tested, the RNase treated, HO stained protocol is the most distinguishable, approaching a single log of separation i.e. ~ minimally a 10 fold increase in fluorescence in infected erythrocytes. However, comparison between SYBR Green and HO stained protocols without RNase treatments, demonstrates that the shift in fluorescence in these protocols is generally greater with corresponding SYBR Green protocols, despite the non-specificity observed with SYBR Green.

In addition to the factors of background non-specific staining and fold separation of infected erythrocytes, all eight protocols were compared to standard Giemsa microscopy based methods to establish accuracy. Comparison of parasitemia values acquired by flow cytometry and Giemsa microscopy from the same culture or dilutions thereof shows positive linear correlation between all methods and the Giemsa method. Correlation coefficients for all methods are equal to or greater than 0.993, with the 5 minute stains being the highest of them all (Fig. 3). Trend lines compared to the expected ideal correlation of $y = x$ all have $m > 1$, where $y= mx + c$. Flow cytometry based methods tested here in this series yield a lower parasitemia than the corresponding parasitemia yielded by the Giemsa stained light microscopic method.

Hoechst staining optimized without permeabilization and RNase treatment

Prior to optimization of HO staining, the standard appearance of 1.5 µM HO in PBS staining of parasite culture for 15 minutes when analyzed by flow cytometry did show a shift, but no significant ‘white space’ between the infected and uninfected erythrocyte populations (1st panel, Fig. 4). Replacing the solvent in which HO is prepared and in which erythrocytes are stained from PBS to RPMI results in a slight increase in separation between infected and

uninfected and presence of a ‘white space’ (Appendix Fig. 2a). Similarly, comparisons of staining protocols performed at room temperature with those performed at 37°C, shows an even more significant improvement in shift of the infected population (Appendix Fig. 2b). HO concentration optimization shows that of the three concentrations tested 1.5, 2 and 3 µM, staining at 2µM results in the cleanest separation (Appendix Fig. 2c). Finally, 1 hour of HO staining has a marginally increased shift in comparison to 15 minutes of HO staining along with a visibly tighter infected erythrocyte population along with a cleaner ‘white space’. The optimized Hoechst method to quantitate infected erythrocytes correlates well with the traditional Giemsa Method ($r^2 = 0.997$).

Fluorescence microscopy of stained samples confirms flow cytometry-based observations

Stained samples visualized under the fluorescence microscope confirm that parasitized erythrocytes are stained. Parasites at different stages of intraerythrocytic growth are stained, by extrapolation of the number of nuclei stained and visualization of erythrocytes in phase showing characteristic haem crystal formation (Fig. 6).

Lipophilic Cell Trackers PKH 26 and PKH 67 detectably stain erythrocytes

PKH 26 and PKH 67 stained and subsequently paraformaldehyde fixed erythrocytes can be visualized under the fluorescence microscope, exhibiting a distinct membrane staining as expected based on their lipophilic properties. Stained erythrocytes can be distinguished from unstained erythrocytes when analyzed on the BD LSR II using the PE and FITC filters for PKH26 and PKH67 cell stains respectively at both 1µM and 2 µM (Fig. 8). Lower concentrations of these dyes, e.g. 0.5 µM have also been shown to distinguishably stain erythrocytes, but these protocols were not thoroughly tested for viability and are therefore not shown here.

Parasites invade PKH 26 and PKH 67 labeled cells at suboptimal efficiency

A simple dual gating strategy was employed to simultaneously visualize flow cytometry measurements of Hoechst staining (x-axis) and PKH cell stain (y-axis) (Fig. 9). This distinguishes the invasion assay sample from an individual well into four subsets of cells: unstained, uninfected erythrocytes (HO –ve, PKH -ve); stained, uninfected erythrocytes (HO-ve, PKH +ve); unstained infected erythrocytes (HO +ve, PKH -ve); stained infected erythrocytes (HO+ve, PKH+ve). No compensation is required because the Hoechst and PKH stain emission spectra are not sufficiently distant from one another. The dissection of cell stains into four quadrants permits comparison of invasion efficiency into stained cells versus unstained cells to be made using three measures: comparison to invasion in another well consisting of parasites incubated with fresh erythrocytes, comparison to invasion in another well consisting of parasites incubated with fresh erythrocytes exposed to diluent treatment i.e. undergoing the staining procedure using buffer without stain, and finally an internal comparison between invasion into stained and unstained erythrocytes within the same well (Table 2). In the last control, invasion efficiency is calculated for a particular PKH stain by first computing the number of HO+ve, PKH+ve erythrocytes as a percentage of the total number of PKH+ve erythrocytes. This is compared to the number of HO+ve, PKH-ve erythrocytes as a percentage of the total number of PKH-ve erythrocytes, thereby obtaining a relative invasion efficiency of invasion into PKH stained cells to PKH unstained cells. This was performed for both PKH 26 and PKH 67 cell stains at 1 µM and 2 µM in both 1:1 and 1:1.25 donor: labeled target cell ratios. Relative invasion rates calculated between well to well comparisons did not uncover any deficiency in invasion into stained cells, except at 2µM concentrations where overall invasion efficiency reduced to 80%. However, internal well comparisons demonstrated that at 2uM invasion

efficiency, in the case of PKH 26, can drop as much to 30% relative invasion efficiency by that internal measure. Using this measure the least toxicity was achieved by using the PKH 26 cell stain at 1μM in a 1:1 donor: stained target cell ratio.

Discussion

Quantification of parasite invasion into erythrocytes

Reported here is the development of a protocol by which parasites can be quantified in a simple two-step procedure involving fixation of the parasites followed by staining of Hoechst 33324 2μM in a total time frame of approximately 2.5 hours, varying by factors that include the number of plates and wells being processed at a given time. The design of the staining procedure in a 96-well format enables multiple invasion assay wells to be processed simultaneously. Assuming that a 96-well plate takes 3 hours to process 96 samples, followed by a 1 hour flow cytometry analysis procedure, conservatively estimated at ~ 30s per well including switching, acquiring over 100,000 cells per well.

In addition to an increase in throughput, the flow cytometry based method preserves the accuracy of the Giemsa microscopy method considered standard in the field, as shown by statistically acceptable correlations. The methods are not in complete agreement due in large part to probable inaccurate counts by the Giemsa method. In general, it appears as though the flow cytometry method estimates a lower parasitemia at small Giemsa measured parasitemia (< 5%). It is important to note that in the comparisons presented here only 1,000 erythrocytes were counted microscopically in the Giemsa method. A more accurate estimation of parasitemia would require a greater number of fields to be counted and other factors such as user expertise to be accounted for by repeating these measurements among several experienced malariologists and numerous slides, to establish gold standard quantification by the Giemsa method.

The thorough analysis of both Hoechst and SYBR Green stains utilizing a variety of protocols involving permeabilization and RNase treatment, to in theory improve the specificity and clean binding of the stains were particularly informative. From these comparisons across

both stains, it appears as though RNase treatment cleans up non-specific binding most apparently in SYBR Green stained samples. RNase treatment may be cleaning up binding of hemoglobin RNA molecules or potentially ribosomal RNA found in reticulocytes in the erythrocyte population. This non-specific binding may be explained by anecdotal observations that SYBR green, which is typically used in polymerase chain reaction (PCR) product staining in agarose gels or real time PCR, is known to exhibit non-specific binding even in these applications (Bengtsson et al., 2003). Hoechst 33324 in contrast has comparatively less background staining that is sensitive to RNase treatment. Hoechst 33324 is dsDNA-specific, binding stoichiometrically to the minor groove and with high affinity to AT containing sequences (Bazhulina et al., 2009). Other than the cleaner results provided by Hoechst staining without permeabilization and RNase treatment, the AT bias of Hoechst is a useful attribute when staining AT rich genome containing *P.falciparum* parasites. Moreover, the shorter wavelength of Hoechst emission in the blue part of the spectrum compared to SYBR Green is a useful attribute of the assay, when attempting to design a multi-parameter flow cytometry application. Hoechst staining could in theory be easily combined with cell stains or fluorescent reporter molecules that emit in the green, yellow and red wavelengths.

PKH stain erythrocyte labeling

In principle, the data distinguishing four distinct subsets of erythrocytes within a well, demonstrates in principle the potential to combine DNA stains and erythrocyte labeling to agents to distinguish donor to target cell invasion from donor to donor cell reinvasion. The demonstration of this principle is necessary, and needs to be improved upon, if high throughput quantification of parasite invasion can avoid the need to enzyme treat the donor erythrocytes in

each invasion assay. Besides saving time, the erythrocyte label approach to performing invasion assays brings up a new internal control by comparing unstained cell and stained cell invasion within the same well. This eliminates to a degree the variation between wells associated with pipetting errors that result in different proportions of parasites within donor erythrocytes in subsequent wells.

However, there are toxicity issues with PKH stains of great concern that will likely delay or make one reconsider the use of these stains with Hoechst staining. Toxicity resulting in 50% relative invasion efficiency into stained cells with respect to unstained cells drastically reduces the dynamic range within which invasion can be measured. This is likely to make most downstream assays or applications lose sensitivity in ability to measure variation.

Potential improvements to the assay

The Hoechst optimized protocol without RNase treatment or permeabilization of erythrocytes has been optimized to maximize separation between infected and uninfected erythrocytes. From the various protocols attempted, the observation that RNase treatment clears traces of background staining could be exploited to further clarify the separation achieved. Permeabilization on the other hand appears to increase background or at least availability to background staining. It may be worth attempting RNase treatment without permeabilization or RNase treatment following milder permeabilization treatments. It must be noted that PKH stains and other cell stains that are lipophilic in nature are extracted with most permeabilization methods. Any subsequent variation to the Hoechst based staining method to quantitate parasite invasion should also consider compatibility with cell stains.

Besides lipophilic stains, various other cell labeling technologies are employed in both in vivo and in vitro applications, most notable of them being the Cell Tracker series of chemical probes. These cell trackers are typically designated to label biologically active cells, taking advantage of intracellular esterase activity to hydrolyze and trap a fluorescent metabolite to label cells. In the protocols employed in this study, parasite samples are fixed due to health and safety procedures. Compatibility with fixation procedures is a key consideration in testing a wider range of cell labeling agents.

Both PKH26 and PKH67 stains appear to show toxic effects with respect to invasion. However, toxicity in terms of invasion may not necessarily mean toxicity to egress, which may permit an alternative strategy to combine PKH cell stains with Hoechst parasite staining. Instead of labeling target cells in an invasion assay, donor cells containing parasites could be labeled at a concentration. Provided that this label concentration does not affect the parasite's ability to egress, one could study invasion of parasites into unlabelled cells. This would have the added advantage of studying a more natural system by virtue of the target cells being unlabelled.

Despite the need for further evaluation of the cell staining protocol to score invasion into specific subsets of cells within a single invasion assay, the currently developed optimized Hoechst staining method can be combined with enzymatic inactivation of donor cells in each well. Neuraminidase/trypsin treatment of the donor cells prevents reinvasion of donor cell parasites into donor cells restricting the Hoechst optimized method to scoring invasion events that are primarily invasion of parasites into target cells.

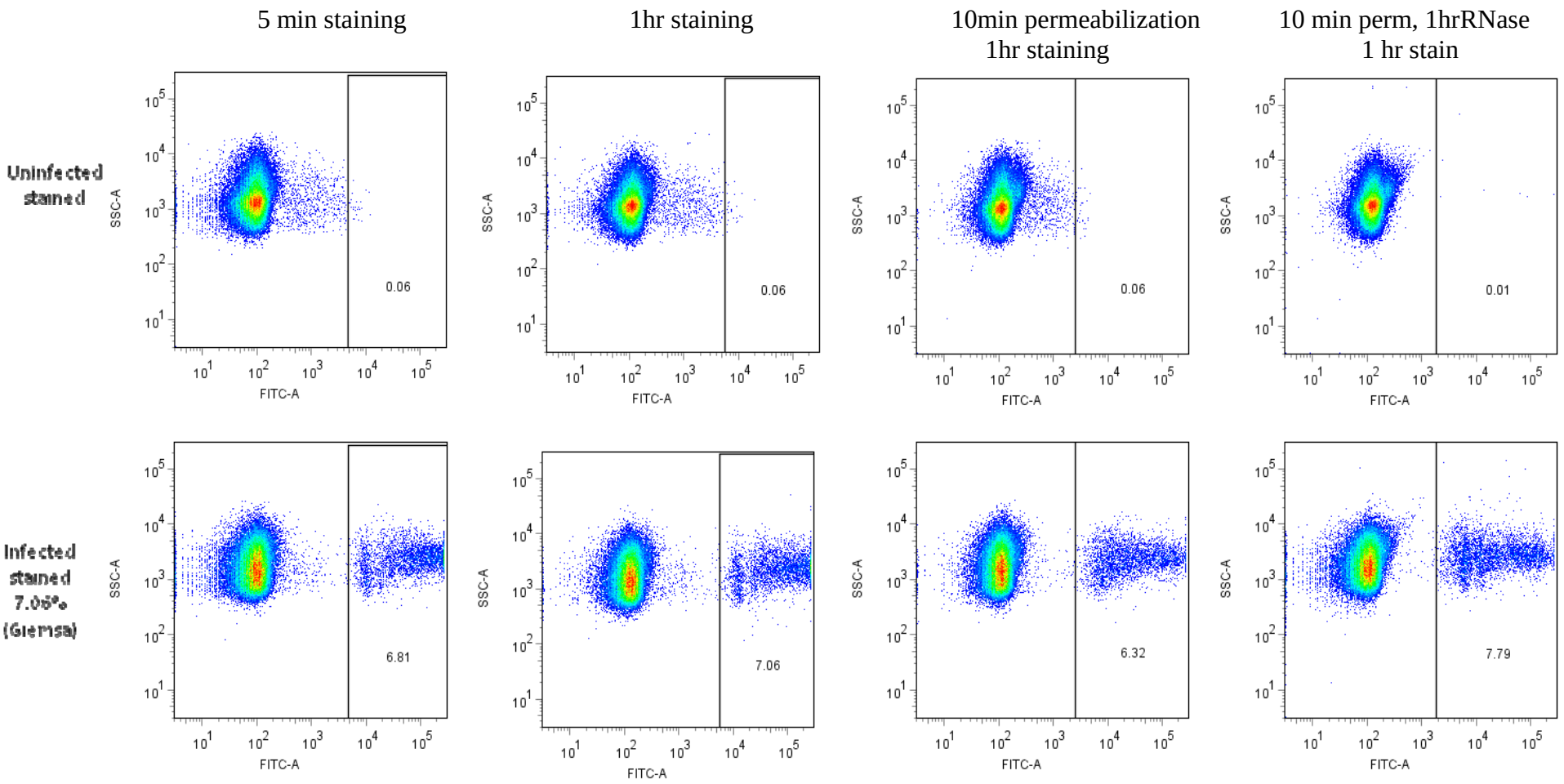
With this basic, high-throughput method, one can begin to explore invasion phenotype variation at a larger scale varying both host and parasite genetics. One immediate application would be to compare invasion phenotypes of a number of field isolates into erythrocytes treated

with different enzyme treatments. The invasion data obtained by the flow cytometry protocol can be combined with whole genome sequencing data of strains as well as functional genomics datasets to understand the molecular basis by which invasion pathway variation patterns are formed. There are however several limitations to what can be accomplished with current sequencing output, in particular the inability to map sequence reads to non-unique regions of the genome.

This kind of high-throughput biological phenotype analysis complemented with genomic approaches could also be extended to the study of specific isolates that exhibit switching from one pathway to another in vitro. Moreover, another interesting experiment would be to keep the parasite genotype constant while varying host genotype by studying invasion into host cells obtained from different individuals. The current Hoechst optimized method could easily be combined with transgenic GFP parasites, perhaps including those that express GFP at particular growth phases in the intraerythrocytic stage and thereby extending information from the assay to include information about post-invasion development of parasites. In combination with rapidly advancing genome sequencing technology, the high-throughput expandable flow cytometry protocol developed here provides a basis upon which we can better understand the molecular mechanisms that underlie host-parasite co-evolution at the invasion interface.

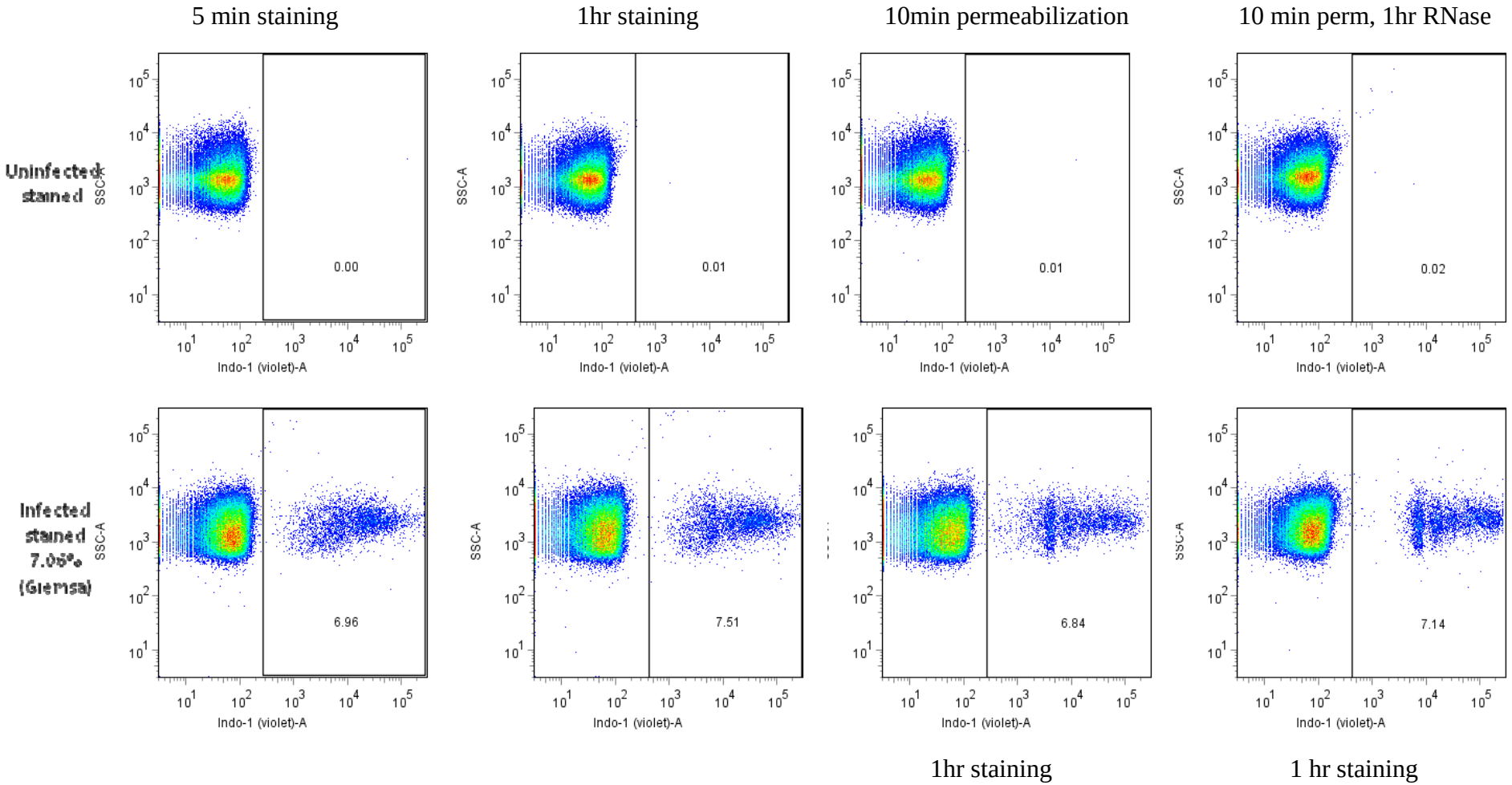
Tables and Figures

Fig. 1: SYBR Green staining of uninfected and infected erythrocytes assessed in a series of conditions



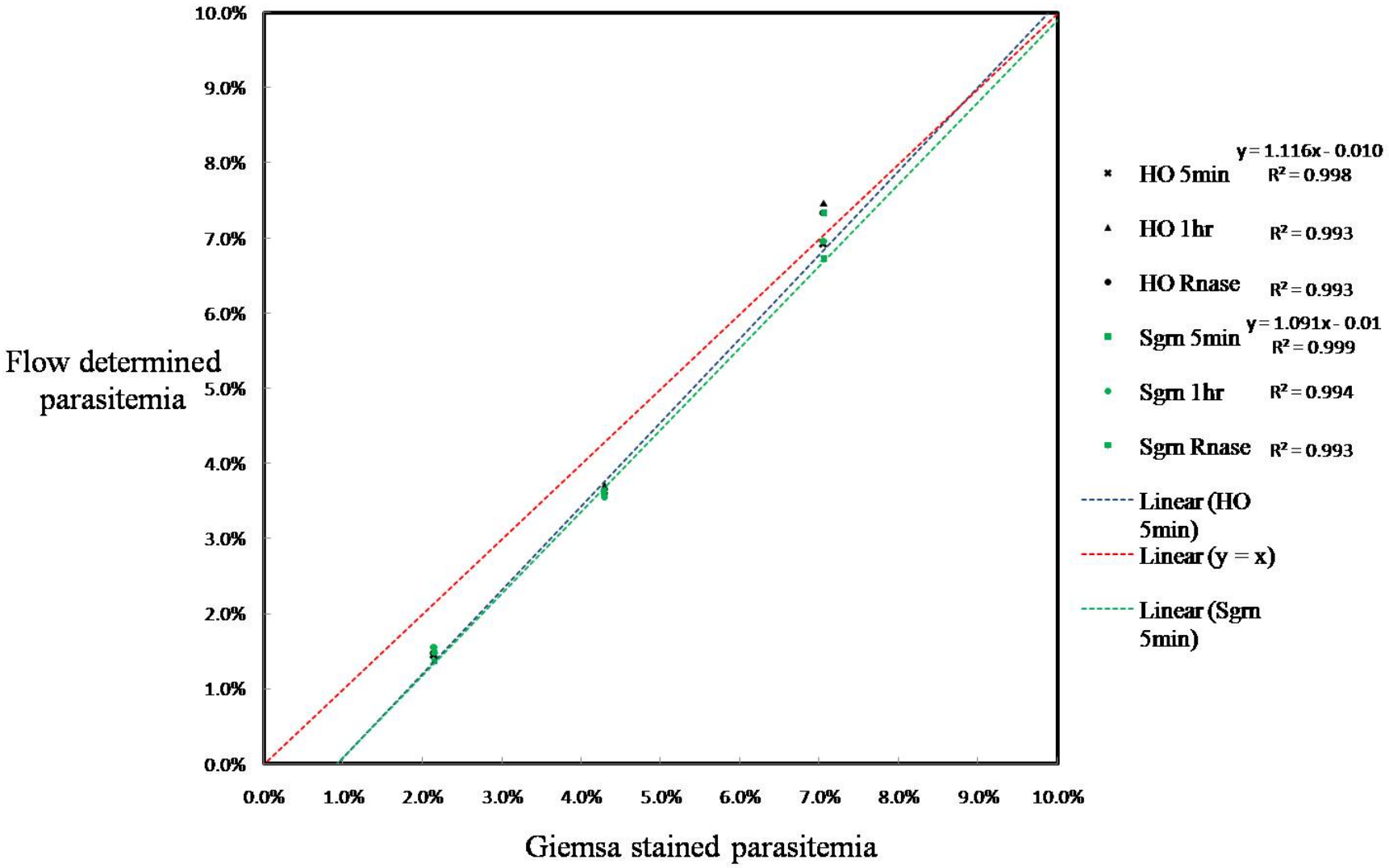
Top row – uninfected erythrocytes stained, bottom row 3D7 infected erythrocytes 7.06% parasitemia according to Giemsa: Wells are 1hr 2% paraformaldehyde 0.1% glutaraldehyde fixed followed by 5 minutes SYBR green stained at 37 ºC; 1 hour SYBR Green stained at 37 ºC; 10 minute 0.3% Triton-X permeabilized at rtp, 1 hour SYBR Green stained at 37 ºC; 10min 0.3% Triton-X permeabilized at rtp, 1hr RNase treatment at 37 °C followed by 1hr SYBR Green staining at 37 °C. Values shown within right box refer to the percentage of cells within the box.

Fig. 2: Hoechst 33324 staining of uninfected and infected erythrocytes assessed in a series of conditions



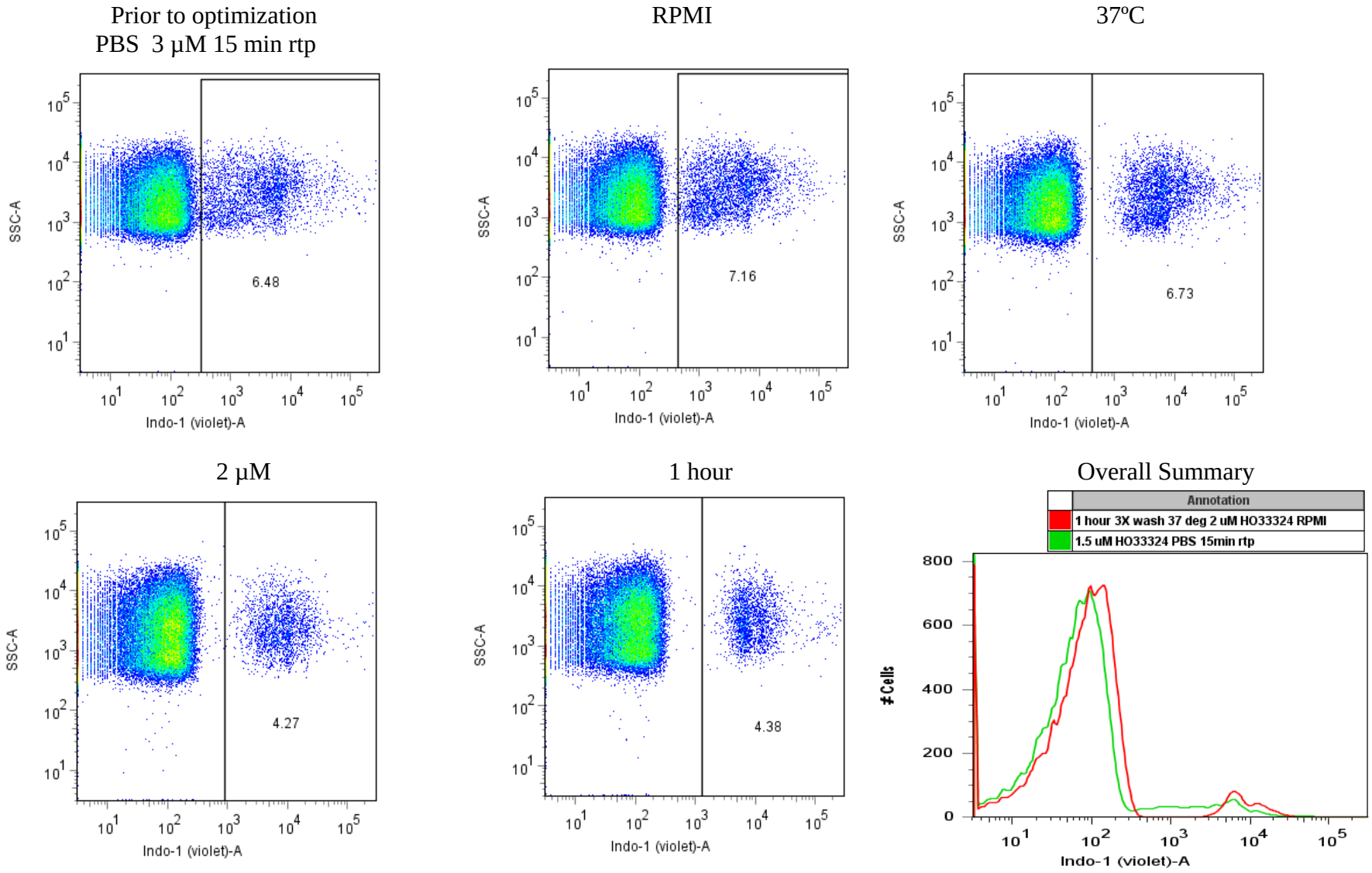
Top row – uninfected erythrocytes stained, bottom row 3D7 infected erythrocytes 7.06% parasitemia according to Giemsa: Wells are 1hr 2% paraformaldehyde 0.1% glutaraldehyde fixed followed by 5 minutes Hoechst stained at 37 ºC; 1 hour Hoechst stained at 37 ºC; 10 minute 0.3% Triton-X permeabilized at rtp, 1 hour Hoechst stained at 37 ºC and finally 10min 0.3% Triton-X permeabilized at rtp, 1hr RNase treatment at 37 °C followed by 1hr Hoechst staining at 37 °C. Values shown within right box refer to the percentage of cells within the box.

Fig. 3: Correlations of Hoechst and SYBR Green quantification with the standard Giemsa protocol



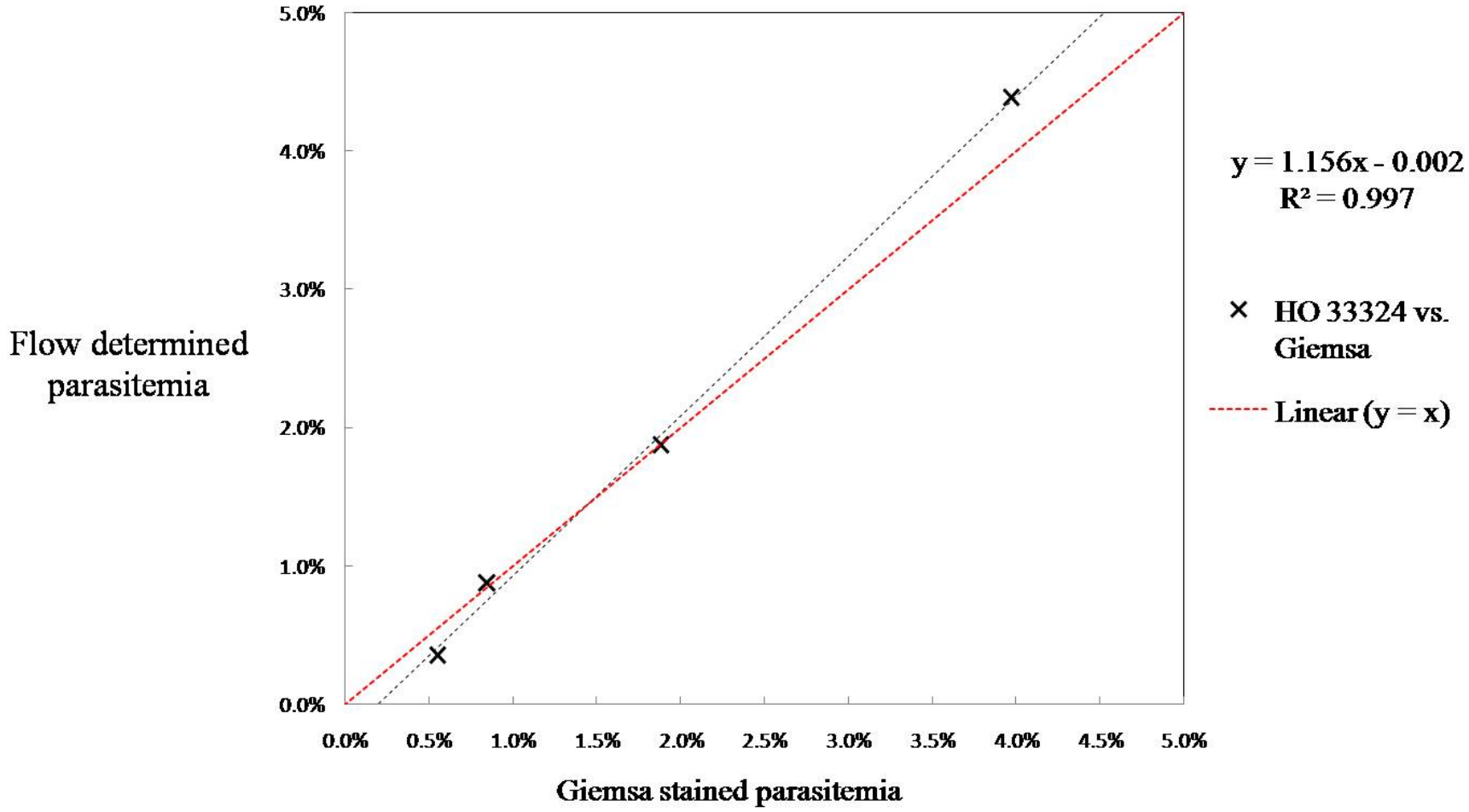
Correlation of flow cytometry determined parasitemia using various staining protocols with parasitemia estimated by microscopy of Giemsa stained parasites. Protocols shown include for both SYBR Green and Hoechst 5 minute staining at 37 ºC; 1 hour staining at 37 ºC; 10 minute 0.3% Triton-X permeabilized at rtp, 1 hour RNase treatment followed by 1 hour staining at 37 ºC

Fig. 4 - Summary of optimization of Hoechst staining without permeabilization and RNase treatments



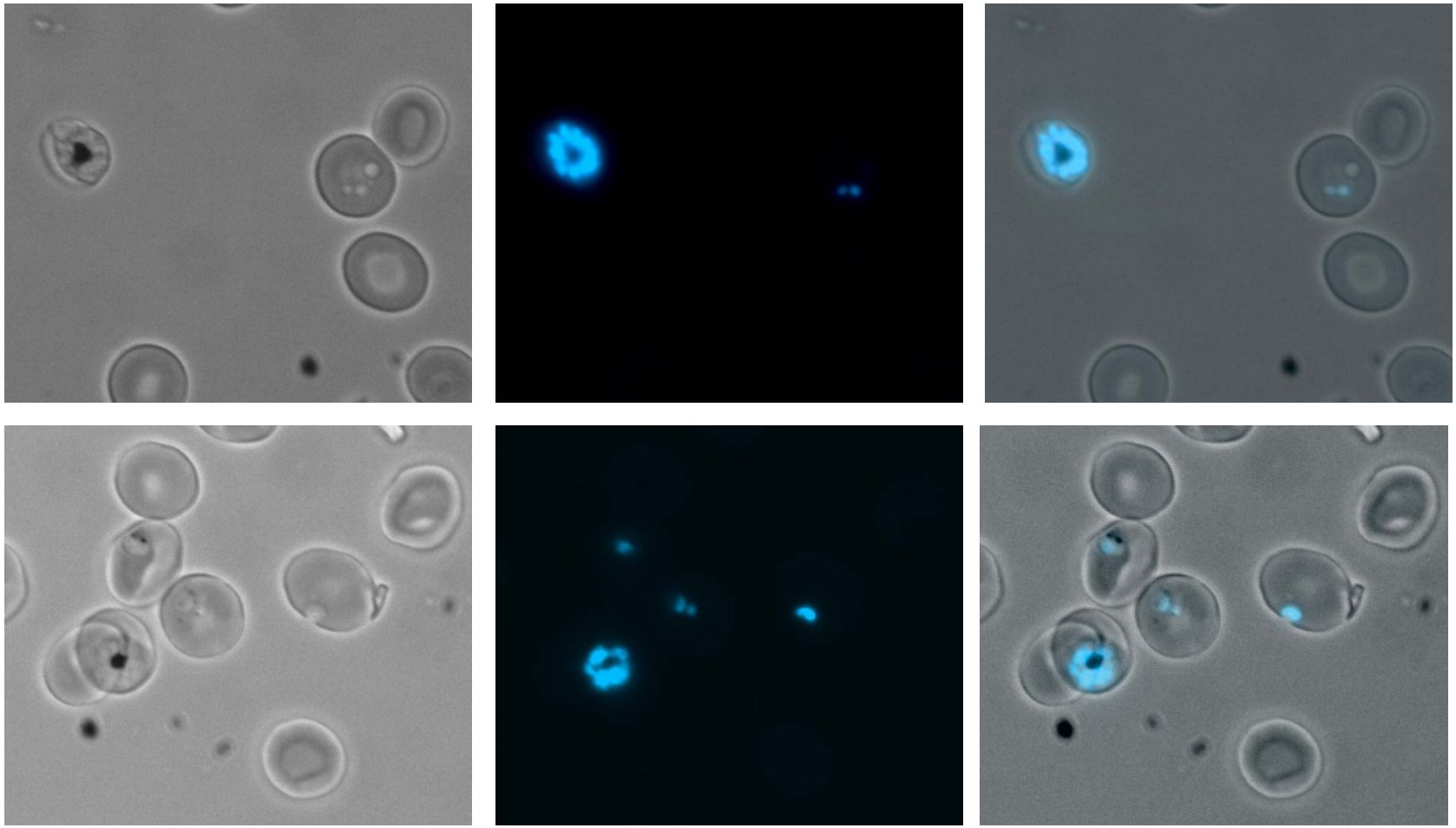
From left to right: (Top) 15 minutes 1.5 uM Hoechst in **PBS** staining at rtp, 15 minutes 1.5 uM Hoechst in **RPMI** staining at rtp, 15 minutes 3 uM Hoechst in RPMI staining at **37 °C** (Bottom) 15 minutes **2 uM** Hoechst in RPMI staining at 37 °C, **1 hour** 2 uM Hoechst in RPMI staining at 37 °C without washing followed by 3X RPMI wash, Overall summary beginning vs. final. Values shown within right box refer to the percentage of cells within the box.

Fig. 5 - Correlation of optimized Hoechst protocol quantification with the standard Giemsa protocol



Correlation of optimized protocol of 1 hour 2 uM Hoechst 33324 dissolved in RPMI staining at 37 °C followed by 3X RPMI wash with parasitemia predicted by counting 1000 Giemsa stained erythrocytes through the light microscope.

Fig. 6 - Hoechst stained parasitized erythrocytes using the optimized protocol visualized under the microscope

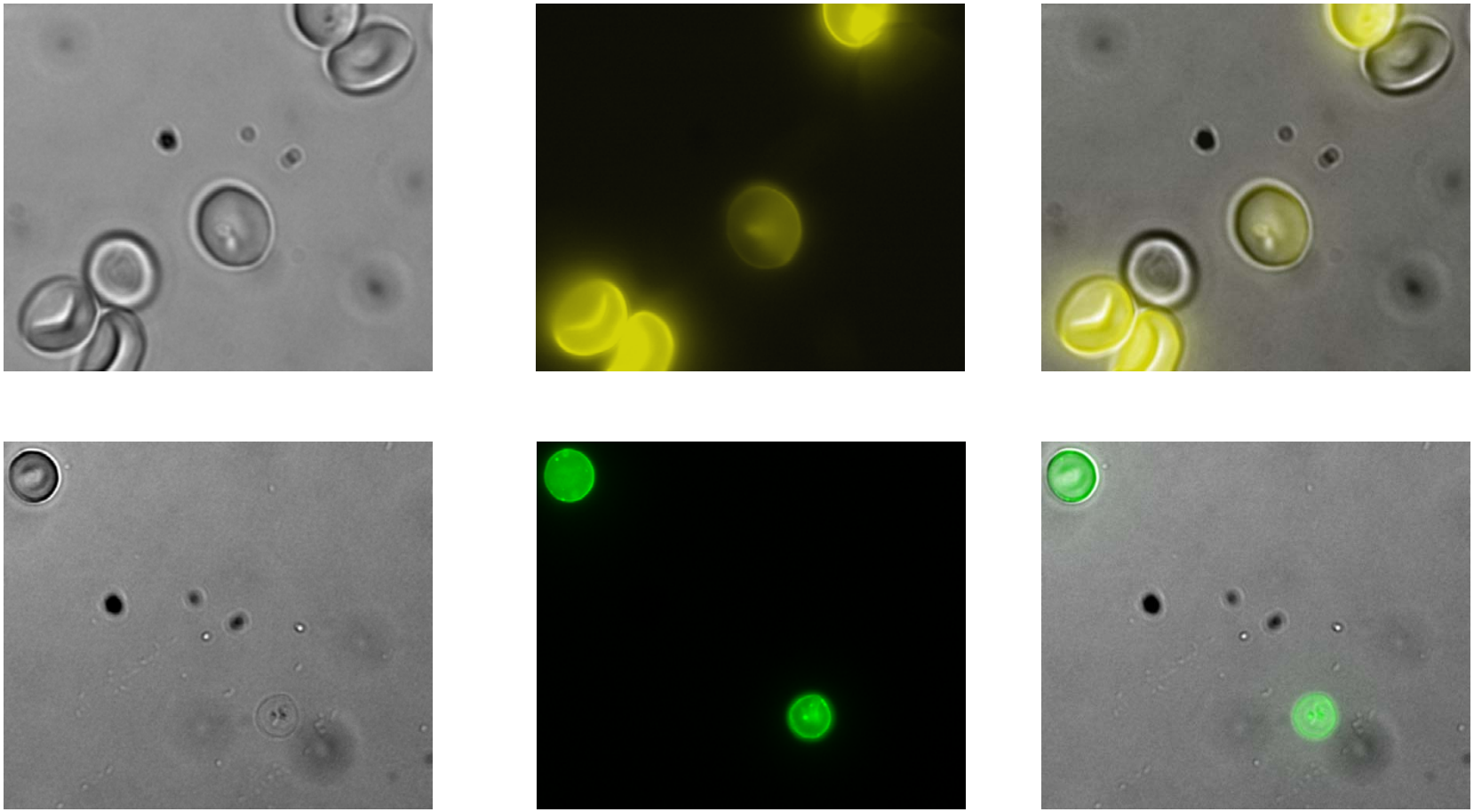


Multiple infected erythrocytes visualized in phase, violet filer and images shown merged.

Table 1 - Comparison of optimized Hoechst protocol with the Giemsa Method

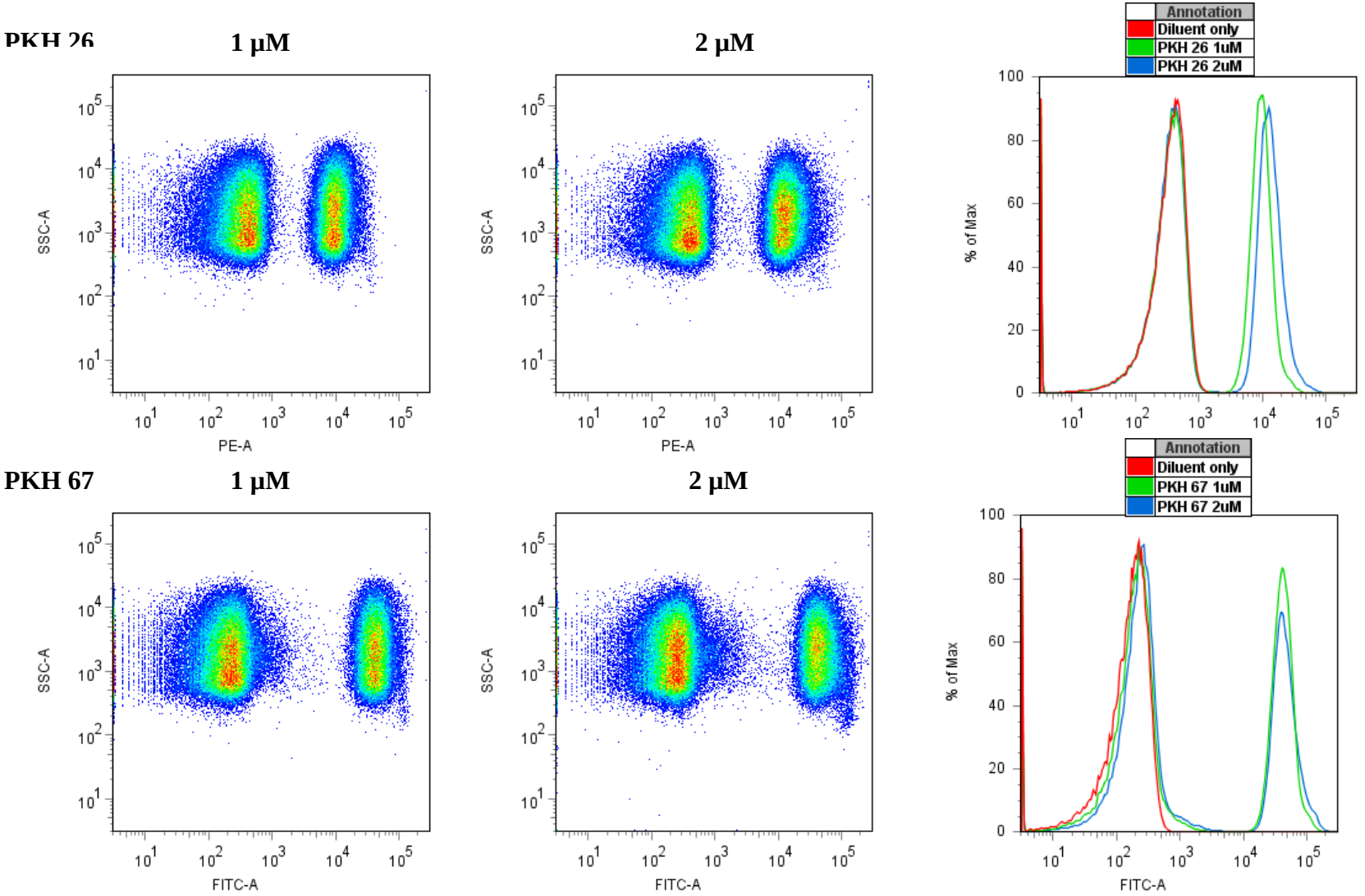
Giemsa Method			Flow Cytometry HO 33324 Method			Absolute Difference
Erythrocytes counted	Infected Erythrocytes	Estimated Parasitemia	Erythrocytes counted	Infected Erythrocytes	Estimated Parasitemia	
1108	44	3.97%	100,000	4392	4.39%	0.42%
1115	21	1.88%	100,000	1745	1.75%	0.13%
1063	9	0.85%	100,000	829	0.83%	0.02%
1086	6	0.55%	100,000	358	0.36%	0.19%

Fig. 7 - PKH 26 and PKH 67 stained erythrocytes visible under the fluorescence microscope



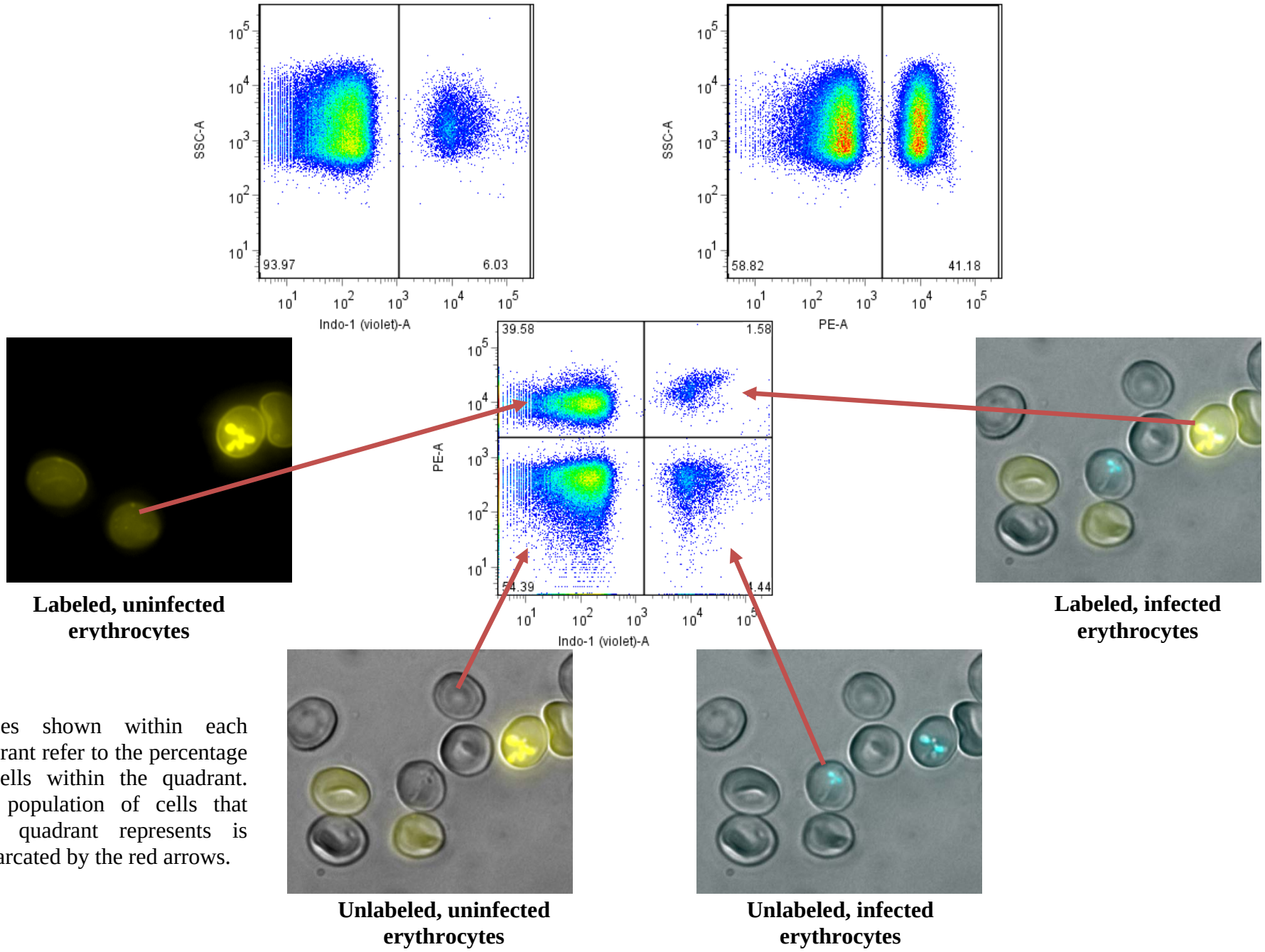
Multiple infected erythrocytes visualized in phase, violet filer and images shown merged 96 hours after staining.

Fig. 8 - Mix of PKH 26 and PKH 67 stained and unstained erythrocytes are distinguished by flow cytometry



Wells composed of a mix of 1:1 donor: stained target cell, showing separation using PKH 26 and PKH 67 dyes over typical 48 hour assay conditions at 37 ºC.

Fig. 9 - Gating strategy used to combine Hoechst staining with PKH erythrocyte stain detection



Values shown within each quadrant refer to the percentage of cells within the quadrant. The population of cells that each quadrant represents is demarcated by the red arrows.

Table 2: Viability Measures of Stained Cells based on relative parasitemia

	Invasion into erythrocytes consisting of a mix of stained and unstained erythrocytes as a % of :					
	Invasion into stained erythrocytes as a % of invasion into unstained cells **within the same well**		Invasion into fresh, unstained erythrocytes treated with **Diluent in another well**		Invasion into fresh, unstained erythrocytes in **another well**	
Ratio of donor: stained target erythrocytes	**1:1**	**1:1.25**	**1:1**	**1:1.25**	**1:1**	**1:1.25**
PKH 26 1 uM	**50.78%**	**46.63%**	**100.50%**	**100.98%**	**105.79%**	**121.56%**
PKH 26 2 uM	**36.84%**	**32.38%**	**92.67%**	**87.20%**	**97.54%**	**104.98%**
PKH 67 1 uM	**45.72%**	**34.50%**	**100.50%**	**109.25%**	**105.79%**	**131.52%**
PKH 67 2 uM	**35.82%**	**31.82%**	**95.67%**	**90.55%**	**100.70%**	**109.00%**

Table comparing invasion efficiency within the same well with invasion efficiency with two controls, the diluent exposed control and the fresh, unstained erythrocytes (diluent unexposed) control

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Appendices

Fig. 1 - Instrumentation

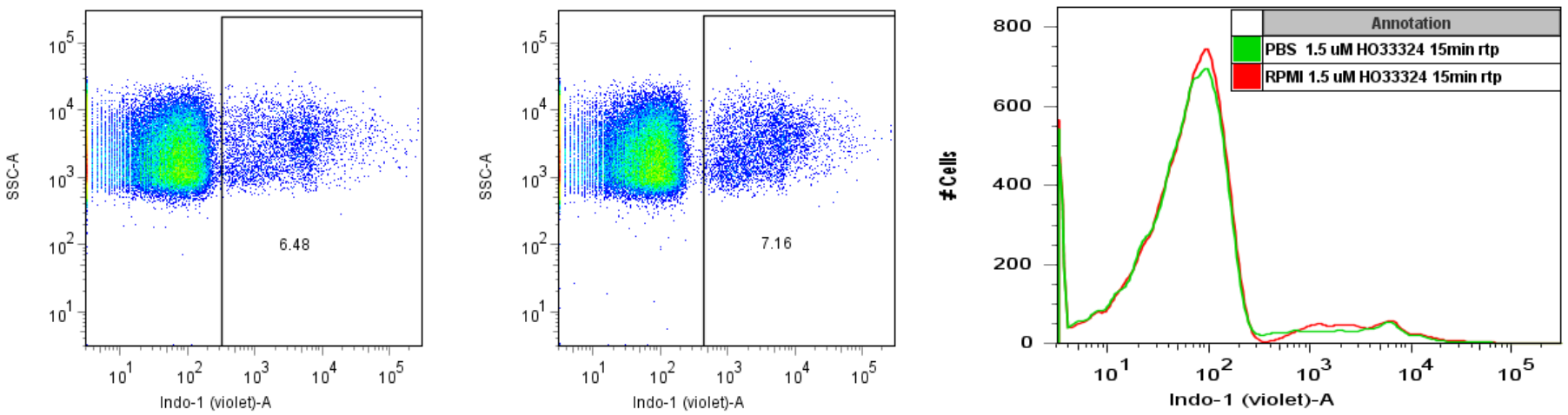
A)

B)

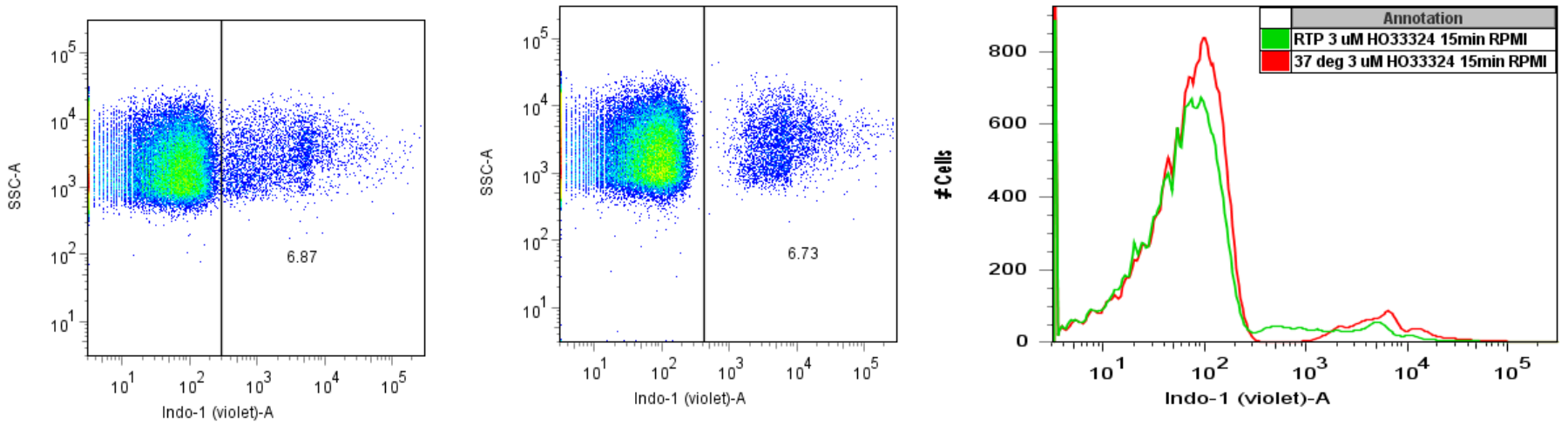
Flow Cytometer Instrumentation: (A) Guava EasyCyte single laser instrument with 96 well plate sampler (Millipore, 2009) (B) BD LSR II four laser instrument with High Throughput Sampler (HTS) 96/384 well plate capability (BD, 2009)

Fig. 2 – Hoechst Protocol Comparisons made during Optimization

(a) PBS RPMI

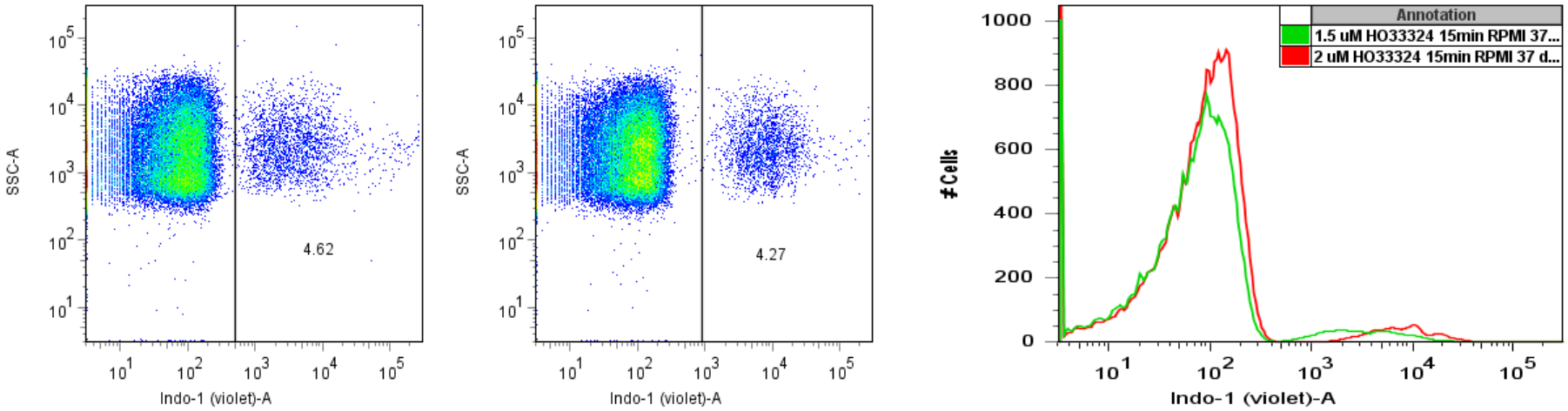


(b) RTP 37°C

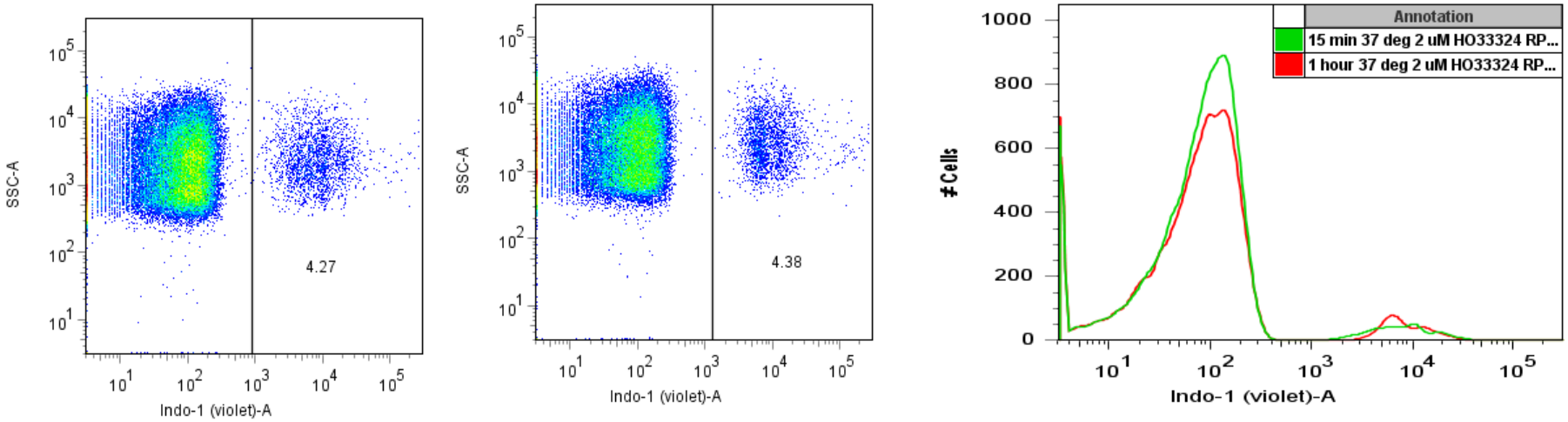


Values shown within right box refer to the percentage of cells within the box.

(c) 1.5 µM 2 µM

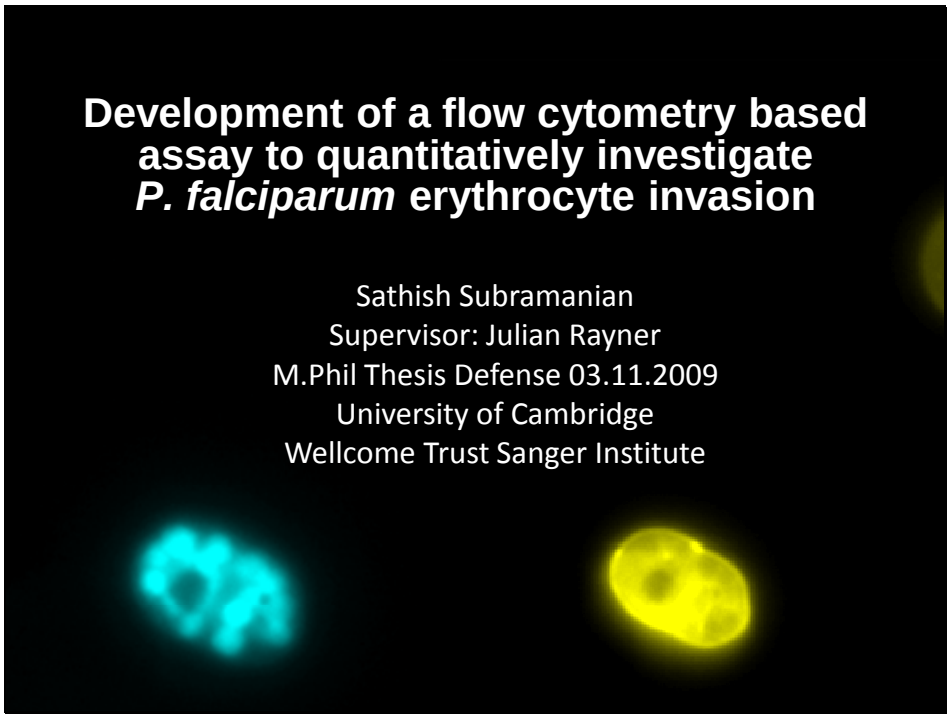


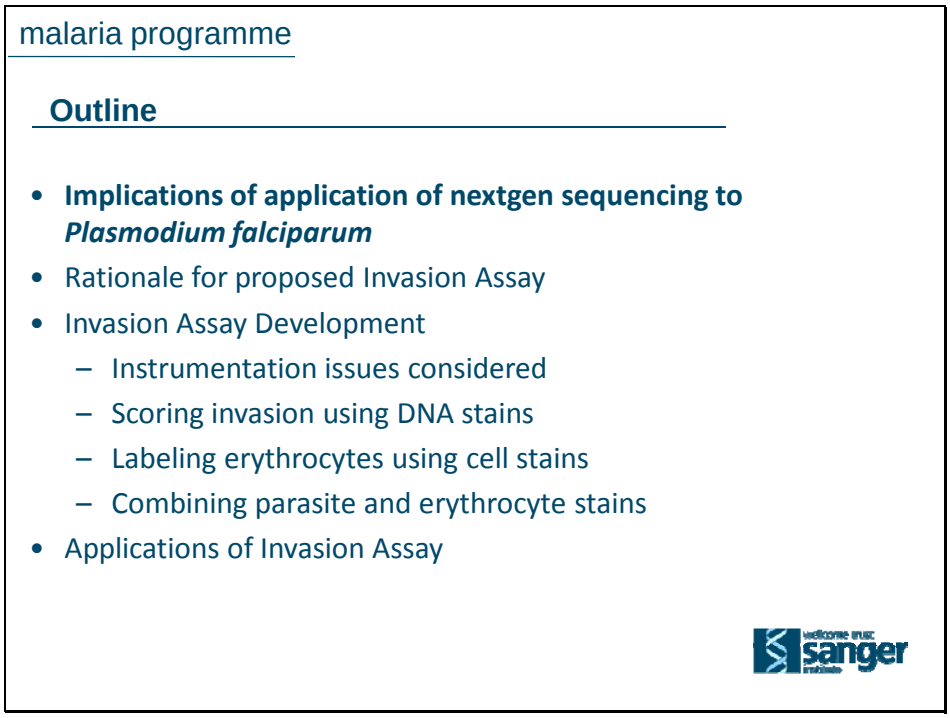
(d) 15 min 1 hour



Values shown within right box refer to the percentage of cells within the box.

Fig. 3 – Attached Presentation from Thesis Defense





malaria programme

Application of nextgen sequencing to *Plasmodium falciparum*

- WTSI contributed to first *Plasmodium falciparum* genome (2002)
- Over the last year, WTSI has been applying Illumina/Solexa sequencing to *P. falciparum,* now over 150 strains sequenced.

Technology	Speed (kbases/hour)	Cost ($/kbase)	Read Length
ABI 3730xl	67	1	700-1000
Roche 454 Titanium	40000	0.1	400
Illumina Genome Analyzer IIx	40000-80000	0.01	35-50
ABI SOLID	> 150000	0.01	35

Preparation/fragmentation of parasite template DNA minimizing human DNA contamination

Ligation of adapters/ purification steps

Base calling with q scores

Mapping of short reads onto 3D7 Reference sequence

SNP, CNV, indel detection software

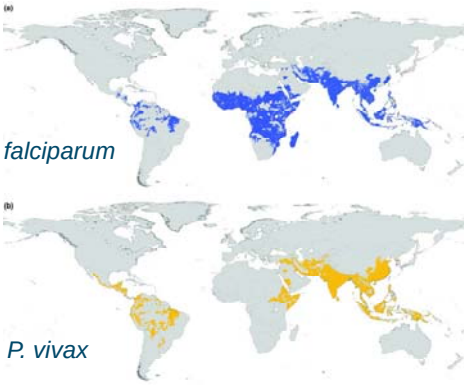
malaria programme

Application of nextgen sequencing to *Plasmodium falciparum*

- Ongoing expansion of sequencing capacity will allow us to profile SNPs, indels, expression levels (RNA-SEQ), ChiP-SEQ for a large number of strains (100s - 1000s) with reduced cost, increased throughput.
- This opens the possibility for GWAS for the first time in *P. falciparum* as well as other hypothesis-generating alternatives to candidate gene approaches by combining Illumina sequencing with phenotyping.
- Phenotypes need to be robust, quantifiable in a high throughput manner, and clinically relevant.
- Options currently limited largely to drug resistance.
- Need for new phenotyping platform to translate this advance in genotyping into advances in understanding *P. falciparum* biology.

malaria programme

Erythrocyte invasion is an important and variable phenotype



P. falciparum

P. vivax

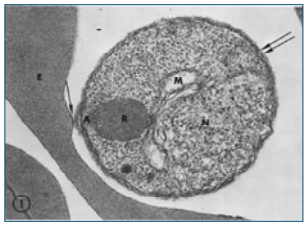
- Essential for parasite survival
- Essential for pathology
- Natural genetic variation known to have major phenotypic effects

- Absence of *P. vivax* in West Africa is due to human genetic variation - Duffy negativity prevents *P. vivax* erythrocyte invasion
- *P. falciparum*? Widespread parasite phenotypic variation in invasion, possible human variation also important

malaria programme

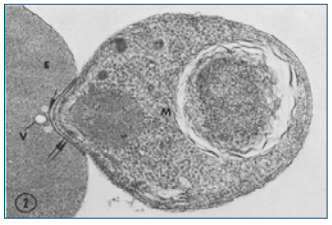
Various host-parasite interactions occur during invasion

Recognition/Apical Attachment



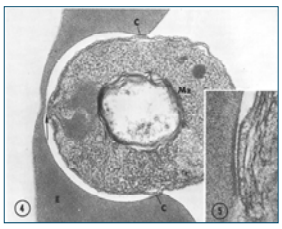
Aikawa *et al.*, J. Exp. Med. 77, 1978.

Junction formation/ RBC deformation

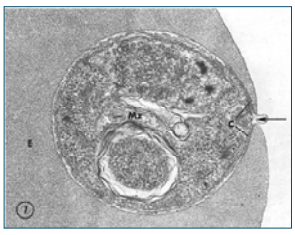


Miller *et al.*, J. Exp. Med. 146, 1979.

Movement of Junction



Invagination & sealing of membrane



malaria programme

Gaps remain in knowledge of molecular players of invasion

- Several parasite ligands/ligand families have been identified:
  - GPI anchored surface proteins (MSP, Pf)
  - Microneme Proteins (AMA-1, EBLs, MTRAP)
  - Rhoptry Proteins
  - Rhoptry Neck Proteins (PfRhs)
- Some RBC receptors identified/proposed using expts with deficient rbcs, mutant parasites, protein-protein interactions and rbc binding assays.
- Host receptor-parasite ligand combination, coined 'invasion pathway', thought to be basis for sensitive/resistant phenotypes with respect to enzyme treatment of RBCs
- *Almost all this evidence comes from experimental manipulation - basis of field phenotypic variation essentially unknown*

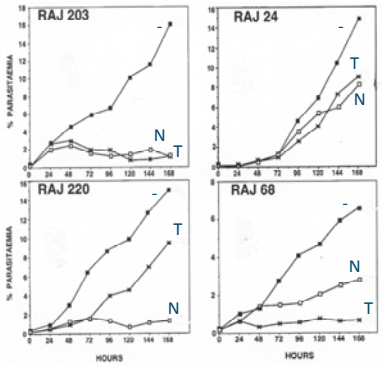
Ligand[a]	Receptor[a]	Sensitivity[b]			Techniques[c]	Refs
		Nm	Tr	ChTr		
EBA175	Glycophorin A	S	S	R	RBCbin, mut, inv and COS	[17,18,36,55]
EBA140[d]	Glycophorin C	S	S	R	RBCbin, mut and overlay	[14–16]
EBA181[d]	? (Receptor E)	S	R	S	RBCbin	[25]
PfRH1	? (Receptor Y)	S	R	R	RBCbin, inv and COS	[21,26,48]
PfRH2a	?	–	–	–		[22,56]
PfRH2b	? (Receptor Z)	R	S/R	S	inv	[22]
PfRH4	?	R	S	S	inv and RBCbin	[19,20,57]
?	? (Receptor X)	R	S	–	inv	[58]
?	Glycophorin B	S	R	S	mut	[58]
?	? (Receptor A)	R	R	S	inv	[47]

Cortés, *(2008) Trends in Parasitology*

malaria programme

Field isolates exhibit invasion pathway variation

- Many groups have shown that different *P.falciparum* field isolates can use different invasion pathways
- Such studies have been limited to relatively small sample sizes (<30)
- Genotype-phenotype correlations limited to analysis of candidate genes only, and all have limited power to detect association b/c of scale



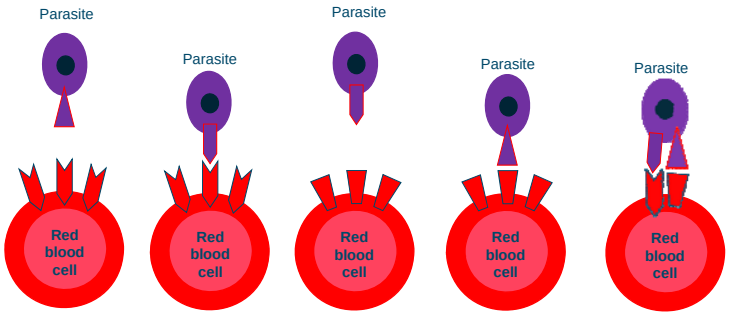
Okoyeh, Pillai and Chitnis, (1999) *Infect. Immun.*

Cortés, *(2008) Trends in Parasitology*

Country and sample size[a]	Technique[b]	Comments	Genes and proteins analyzed	Correlations between ligands[c] Positive	Correlations between ligands[c] Negative	Correlations with invasion pathway[d]	Refs
Kenya (42)	qRT–PCR	*eba-175* predominant in most isolates, *PfRh4* shows greater variability	*eba-175*, *PfRh1*, *PfRh2a*, *PfRh2b* and *PfRh4*	*PfRh2a–PfRh2b*	*eba-175–PfRh2b* *eba-175–PfRh4* *PfRh1–PfRh2b* *PfRh2a–PfRh4*	*eba-175*-Nm sensitive	[43]
Tanzania (11)	Western blot	Complete silencing of some ligands in some isolates	EBA-175, EBA-140, EBA-181, PfRH1, PfRH2a and PfRH2b	PfRH2a–PfRH2b EBA-140–EBA-175 EBA-181–PfRH2b	PfRH1–PfRH2a PfRH1–PfRH2b	PfRH1-Tr and ChTr resistant PfRH2a, PfRH2b and EBA-181-Tr and ChTr sensitive	[45]
Senegal (12)	Western blot	Minimal differences in expression	PfRH1, PfRH2a and PfRH2b				[46]

malaria programme

Invasion pathway variation: influenced by parasite, host or both?



Overarching goal in studying invasion pathway variation phenotypes:

- Elucidate identities and roles of host and parasite molecules involved in invasion not know to date
- Explore in vivo significance of invasion pathway variation
- Inform therapeutic approaches targeted at blocking invasion components

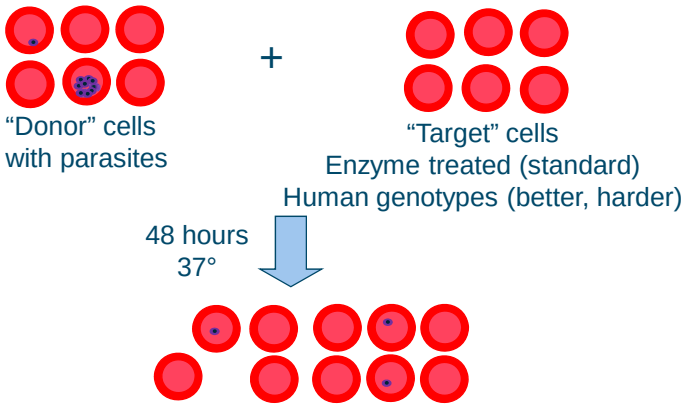
malaria programme

Outline

- Implications of application of nextgen sequencing to *Plasmodium falciparum*
- **Rationale for proposed erythrocyte invasion assay**
- Invasion Assay Development
  - Instrumentation issues considered
  - Scoring Invasion using DNA stains
  - Labeling Erythrocytes using cell stains
  - Combining parasite and erythrocyte stains
- Applications of Assay

malaria programme

Invasion phenotyping is a two-fold problem



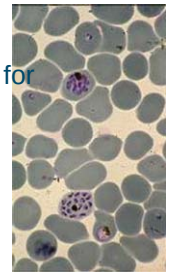
Challenges:
1) Counting parasites – Scale
2) Distinguishing invasion into target from donor cells
   - Methodological

malaria programme

Invasion phenotyping is a two-fold problem

Traditional Invasion Assays use a combination of enzyme treatments or purification steps along with a Giemsa Stain followed by microscopy

- To prevent reinvasion into donor cells, studies either purify late stage parasites (**not trivial**) or pre-treat donor cells (**not completely effective**)
- Mix with target erythrocytes (often enzyme treated), incubate for 24 hours, smear, Giemsa stain and count by microscopy.



Disadvantages:

- Limited by **scale and user expertise** which could lead to **inconsistency** across multiple users
- **Low throughput** impedes progress of a large scale project working with numerous strains
- Have to enzyme inactivate donor erythrocytes or purify late stage parasites to score donor to target rbc invasion

(Laborious and also a source of inconsistency)

malaria programme

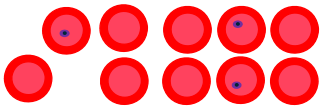
Invasion phenotyping is a two-fold problem: scalability

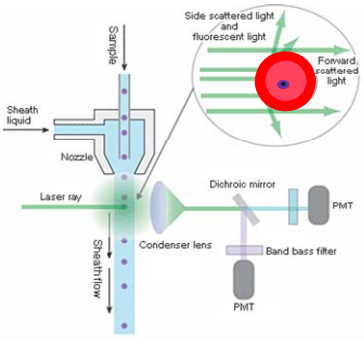
"Donor" cells
with parasites

+

"Target" cells
Enzyme treated (standard)
Human genotypes (better, harder)

48 hours
37°

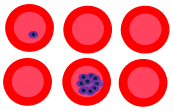




Challenges:
1) **Counting parasites - Scale**
2) Distinguishing invasion into target & donor cells - Methodological
Solution: **Multi-Parameter Flow cytometry**

malaria programme

Invasion phenotyping is a two-fold problem : methodological



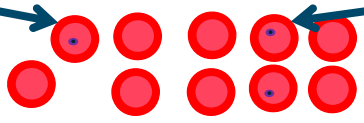
"Donor" cells
with parasites

+

"Target" cells
Enzyme treated (standard)
Human genotypes (better, harder)

48 hours
37°

Invasion into Donor Cell



Invasion into Target Cell

These two types of invasion events are indistinguishable by Giemsa-stained microscopy without either elimination of donor cells by enzyme inactivation or purification of late stage parasites.

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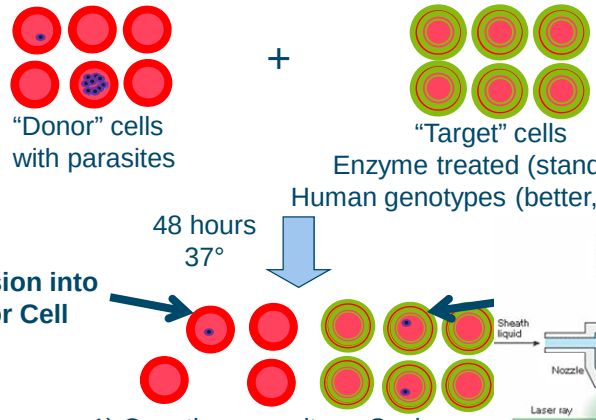
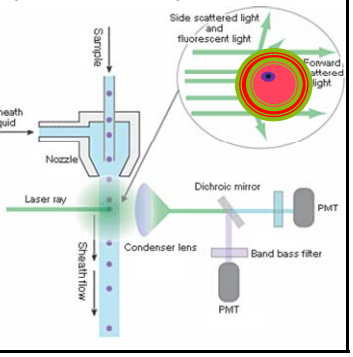
malaria programme

Invasion phenotyping is a two-fold problem: methodological



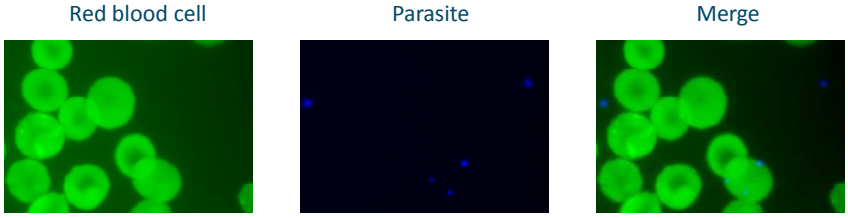


Challenges: 1) Counting parasites - Scale
2) **Distinguishing target and dor**
Methodological

Solution: **Multi-Parameter Flow cytometry**

malaria programme

Proposed Assay combines cell and parasite labelling



Using **fluorescently labeled red blood cells and parasites** to develop a new high throughput assay to measure invasion events by **flow cytometry**.

Output: able to measure impact of **natural and experimental genetic variation** in both host and parasite on red blood cell invasion

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malaria programme

Outline

- Implications of application of nextgen sequencing to *Plasmodium falciparum*
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  - **Instrumentation issues considered**
  - **Scoring Invasion using DNA stains**
  - Labeling Erythrocytes using cell stains
  - Combining parasite and erythrocyte stains
- Applications of Assay

malaria programme

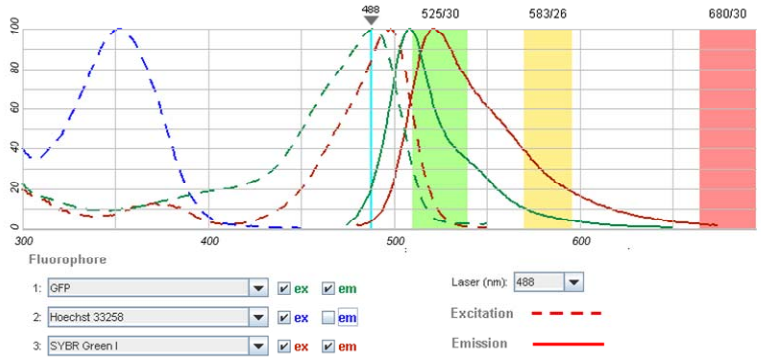
Flow Cytometry Instrumentation available has tradeoffs

- **Guava:**
  - Single laser (488nm), upto 3 colour
  - **Contained within CL3 facility**
  - **Cheap, compact and portable**
  - 96 well plate format, low-medium throughput (~ 400 cells/s)

- **BD LSR II**
  - Four laser (355nm, 405 nm, 488nm, 633 nm), upto 18 colour
  - **Contained in CL1 facility, requires extra health & safety approval**
  - Expensive (though core facility)
  - 96 well plate format, **ultra-high throughput** (~ 6000 cells/s)
  - More **sensitive** instrument

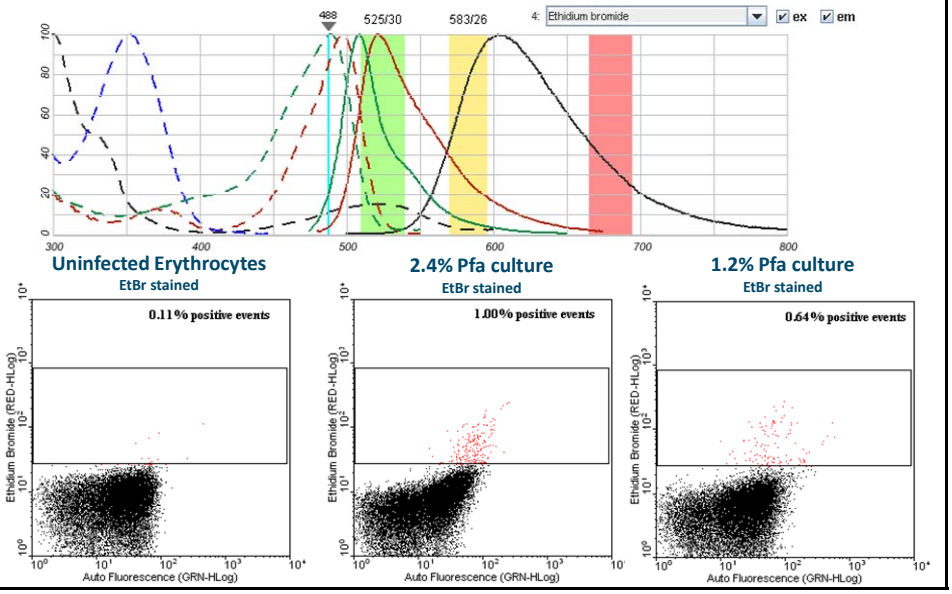
malaria programme

Guava 488nm laser restricts range of dyes that can be tested



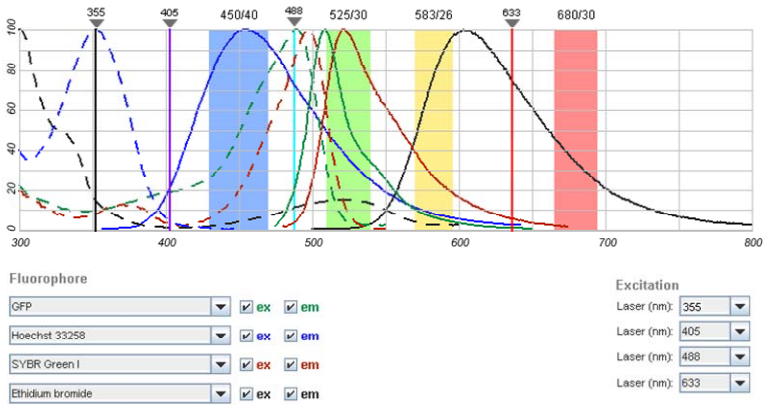
malaria programme

Guava data showed insufficient separation and throughput



malaria programme

BD LSR II enables rapid and wider range of fluorochrome testing



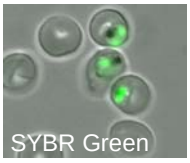
Four laser, high throughput instrument, suitable for multiparameter applications:
e.g. Hoechst DNA stain, GFP (transgenic strain in the future), Red RBC label
One caveat: H&S Approval July 2009
All experiments presented here and in thesis were performed using BD LSR II

malaria programme

Scoring invasion events using DNA dyes

DNA dye selection considerations:
DNA/RNA specificity, Cell Permeability, AT bias *P.falciparum* genome
Tested as follows with dyes **Hoechst 33324** and **SYBR Green**:

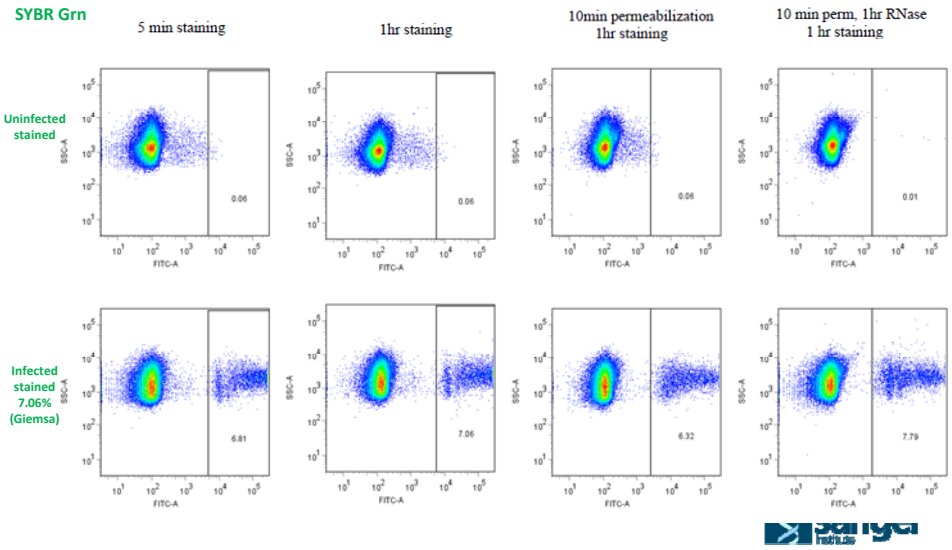
Fixation of RBCs
↓
DNA Staining ←
↓
Triton-X permeabilization
↓
DNA Staining ←
↓
RNAse treatment
↓
DNA Staining (high level of Hb RNA) ←



Data Output considerations:
Separation of infected and uninfected, consistent calling of positives independent of the user, correlation with existing Giemsa microscope method and compatibility with cell staining protocols

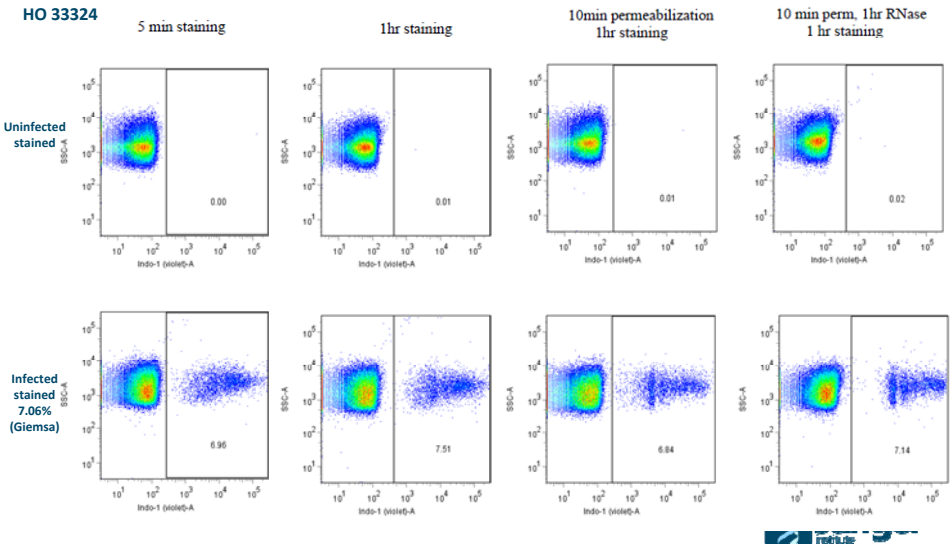
malaria programme

SYBR Green staining assessed in a series of conditions



malaria programme

Hoechst 33324 3uM staining assessed in a series of conditions



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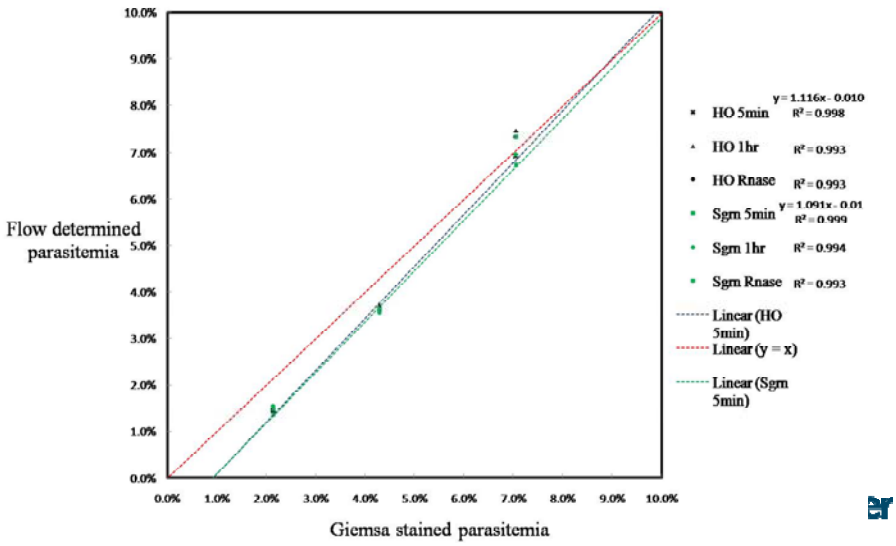
Correlations with traditional Giemsa method



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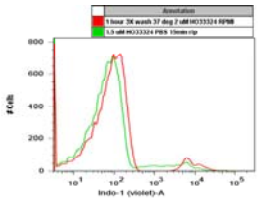
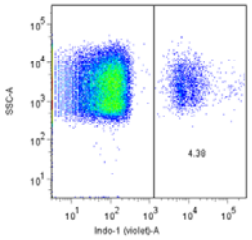
HO 33324 staining without permeabilization & RNase steps

- HO 33324 appears to be cleaner than SYBR Green and is cell permeant
- HO 33324 is unlikely to conflict with the use of other fluorochromes with its emission being restricted to the blue range of the spectra
- Scoring invasion with a DNA dye protocol that avoids permeabilization is compatible with a wider range of cell labelling technologies and avoids RNAse treatment.
- Staining Buffer, Staining Temperature, Dye Concentration, Time of staining, Post-staining washing were optimized
- 2 µM HO 33324 staining in RPMI at 37 °C for 1 hour followed by 3X wash is the protocol that most distinguishes infected erythrocytes

Summary of HO 33324 optimization



2 µM HO 33324 1hr 37°C



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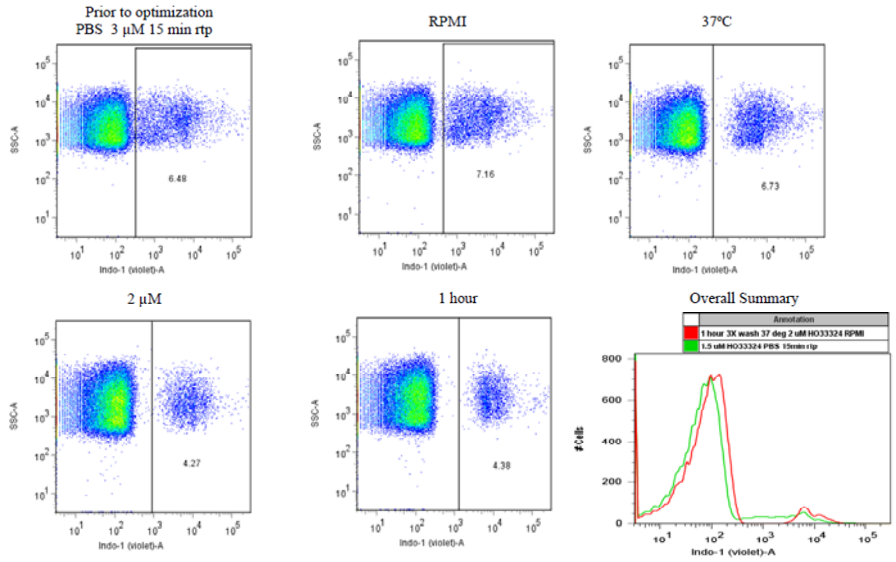
HO 33324 staining without Perm/RNAse optimization steps



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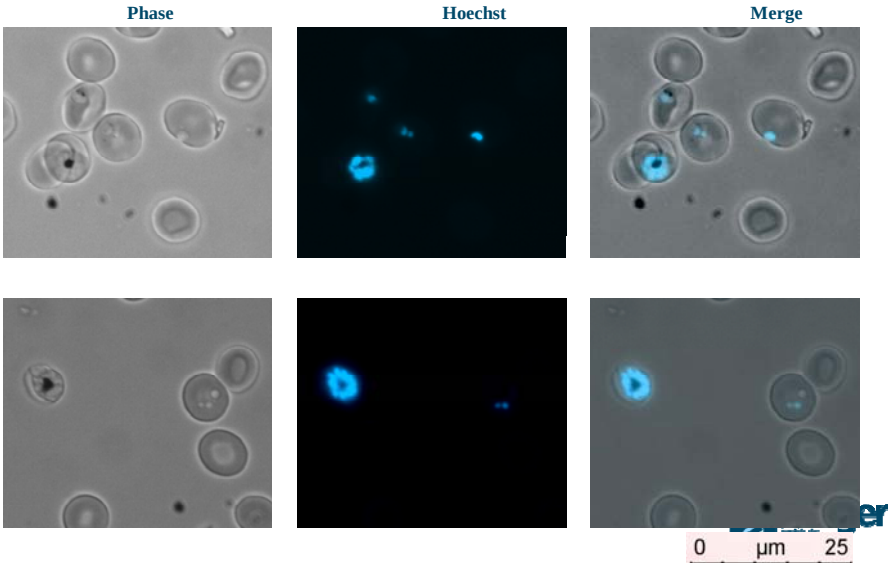
Hoechst stained parasites under fluorescence microscope



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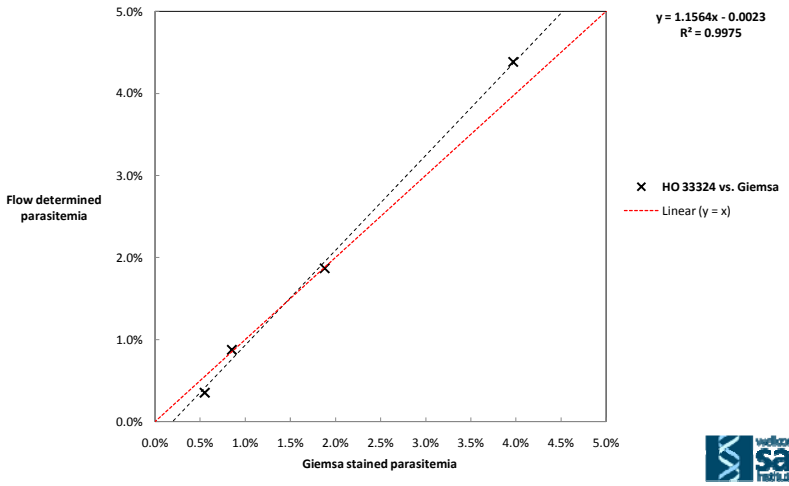
Optimized Hoechst staining method correlates with Giemsa



malaria programme

Comparison Table of Optimized Hoechst 33324 staining method and Giemsa method

Giemsa Method			Flow Cytometry HO 33324 Method			Absolute Difference
Erythrocytes counted	Infected Erythrocytes	Estimated Parasitemia	Erythrocytes counted	Infected Erythrocytes	Estimated Parasitemia	
1108	44	3.97%	100,000	4392	4.39%	0.42%
1115	21	1.88%	100,000	1745	1.75%	0.13%
1063	9	0.85%	100,000	829	0.83%	0.02%
1086	6	0.55%	100,000	358	0.36%	0.19%

malaria programme

Outline

- Implications of application of nextgen sequencing to *Plasmodium falciparum*
- Rationale for proposed Invasion Assay
- **Invasion Assay Development**
  - Instrumentation issues considered
  - Scoring invasion using DNA stains
  - **Labeling erythrocytes using cell stains**
  - **Combining parasite and erythrocyte stains**
- Applications of Invasion Assay

malaria programme

Erythrocyte Labelling

Label selection considerations:

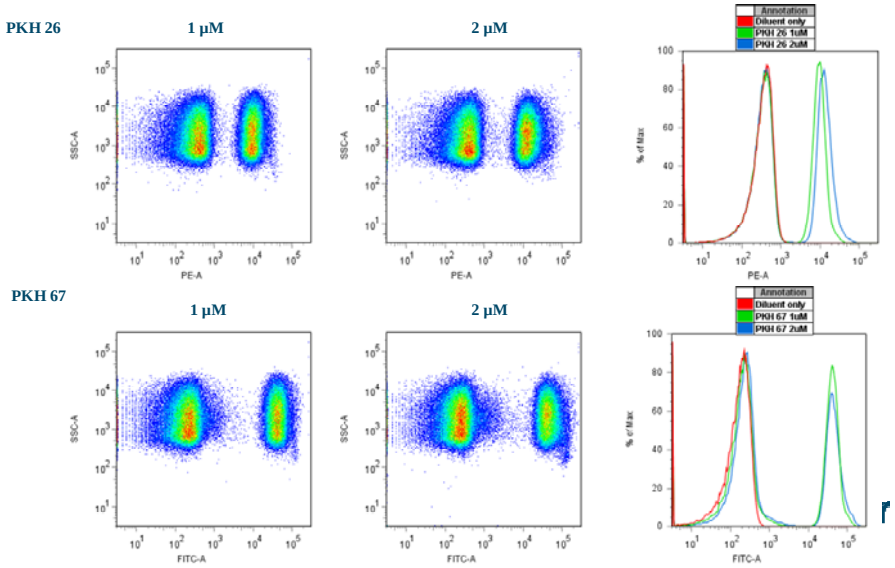
- Labels RBCs detectably, stable for over 48 hours, avoids leakiness, toxicity and/or perturbation of invasion biology

PKH dyes general membrane labelling dyes

- PKH (lipophillic dyes) used with mouse rbcs for in vivo applications ranging upto 6-100 days.
- PKH 67 and PKH 26 differ in their emission spectra green and red respectively, length of lipid chain, but both are compatible with optimized Hoechst protocol.
- Labelling procedures involves brief incubation of cells in a medium without salt called diluent followed by several wash steps.
- Dye concentration and time of labelling are yet to be fully optimized

malaria programme

PKH 26/PKH 67 stained cells mixed and incubated with unstained cells distinguished by flow cytometry



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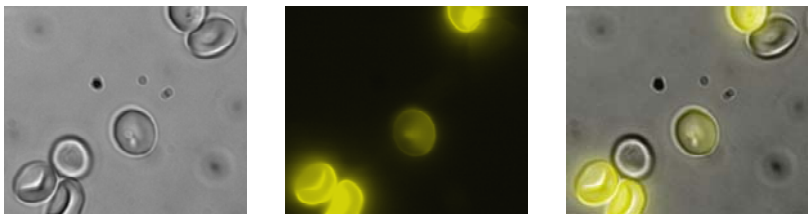
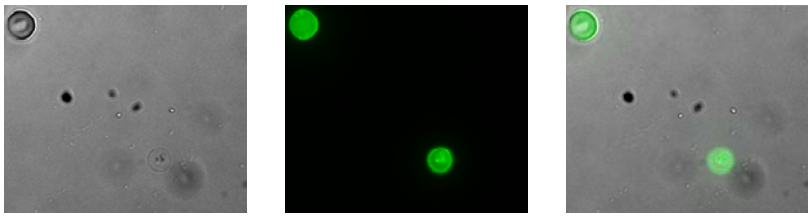
PKH labelled cells distinguished under the fluorescence microscope

PKH 26 (PE Channel)



PKH 67 (FITC Channel)



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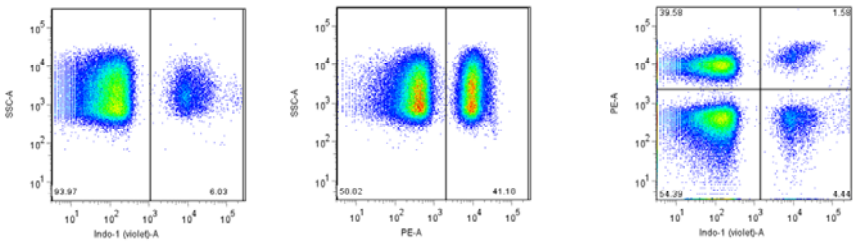
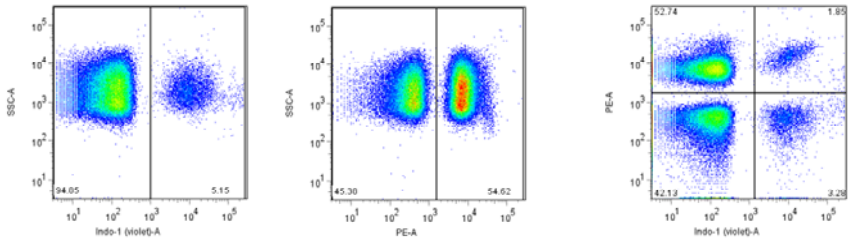
Combining parasite staining with erythrocyte staining

HO 33324 optimized stain + PKH 26 1µM → HO 33324 / PKH 26

1:1 donor : labelled target

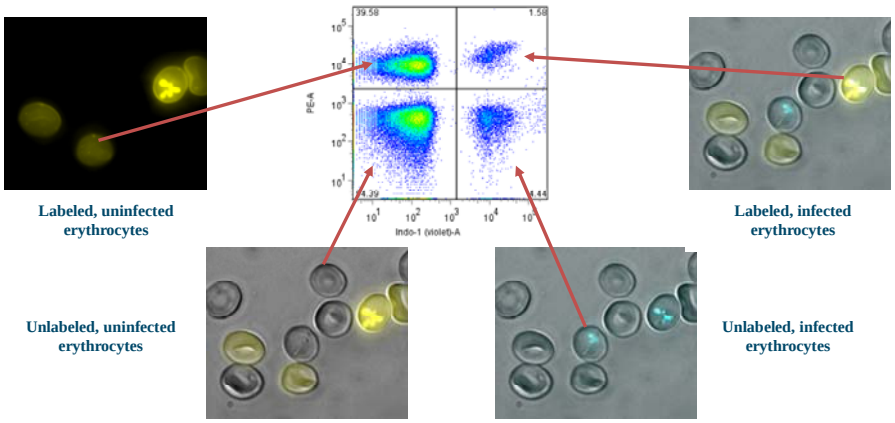


1:1.25 donor : labelled target



malaria programme

Double gating enables calculation of an internal stained cell population parasitemia



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PKH 26/67 staining of target cells has subtle effects on overall Invasion efficiency, but clearly reduces stained cell-specific invasion efficiency

			Invasion into erythrocytes consisting of a mix of stained and unstained erythrocytes as a % of :			
	Invasion into stained erythrocytes as a % of invasion into unstained cells **within the same well**		Invasion into fresh, unstained erythrocytes treated with **Diluent in another well**		Invasion into fresh, unstained erythrocytes in **another well**	
Ratio of donor: stained target erythrocytes	**1:1**	**1:1.25**	**1:1**	**1:1.25**	**1:1**	**1:1.25**
PKH 26 1 uM	**50.78%**	**46.63%**	**100.50%**	**100.98%**	**105.79%**	**121.56%**
PKH 26 2 uM	**36.84%**	**32.38%**	**92.67%**	**87.20%**	**97.54%**	**104.98%**
PKH 67 1 uM	**45.72%**	**34.50%**	**100.50%**	**109.25%**	**105.79%**	**131.52%**
PKH 67 2 uM	**35.82%**	**31.82%**	**95.67%**	**90.55%**	**100.70%**	**109.00%**

malaria programme

Summary of Invasion Assay Development

Assay Goals Achieved:

- Optimized Hoechst method correlates with existing Giemsa method, improving on **scalability**
- Shown labelling of rbcs by one family of lipophilic dyes and detection by flow cytometry
- Combined cell labels with optimized Hoechst staining method to show as **proof of principle to distinguish invasion into target cells using flow cytometry**, albeit with definite toxicity issues that need to be addressed.

Alternative approaches with PKH dyes

- PKH dye toxicity may be reduced by lowering stain concentration
- If PKH dyes are toxic, the reverse experiment in which donor cells are labelled and target cells are unlabelled could be pursued.

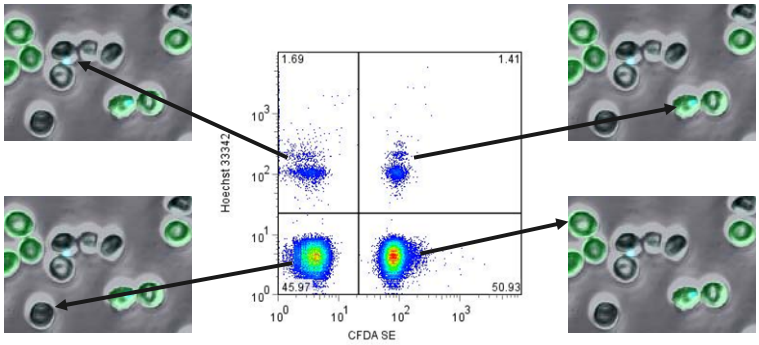
Other Cell labelling agents

- CFDA-SE, CellTracker Agents

malaria programme

Summary of follow-up work in lab not reported in thesis

Combining CFDA-SE + Optimize Hoechst Staining Method

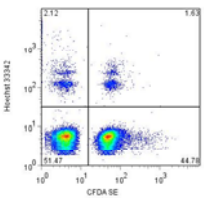


Credit: Michel Theron

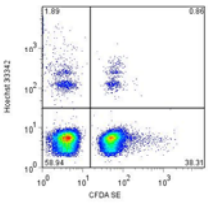
malaria programme

Invasion pathway phenotype of known laboratory strains reproduced

3D7 – Nm Resistant Strain

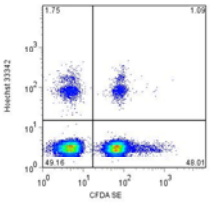


Untreated

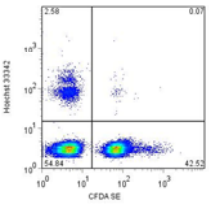


Nm treated

Dd2 – Nm sensitive strain



Untreated



Nm treated

Credit: Michel Theron

malaria programme

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  - Labeling Erythrocytes using cell stains
  - Combining parasite and erythrocyte stains
- **Applications of Assay**

malaria programme

Invasion phenotyping: Applications

- **Applications to natural genetic variation:**
  - **A) Parasite variation**
    - *In vitro* adapted lines (field isolates, progeny from genetic crosses)
    - *Ex vivo* assays in the field straight from the arm (culture parasites to schizont stage, add to labeled RBCs, count 24 hours later)
    - Combine with Illumina DNA/RNA seq to perform GWAS/hypothesis generation
  - **B) Host variation**
    - Label erythrocytes from genotyped or blood typed individuals
    - Both field approaches facilitated by links to MalariaGEN Consortium of researchers in 21 endemic countries
- **Applications to experimental genetic variation:**
  - Same assay can be applied to measure inhibition of invasion *in vitro,* such as by antibodies, recombinant proteins, compounds or libraries
  - Similarly, can be used to compare knockout and wildtype parasite lines

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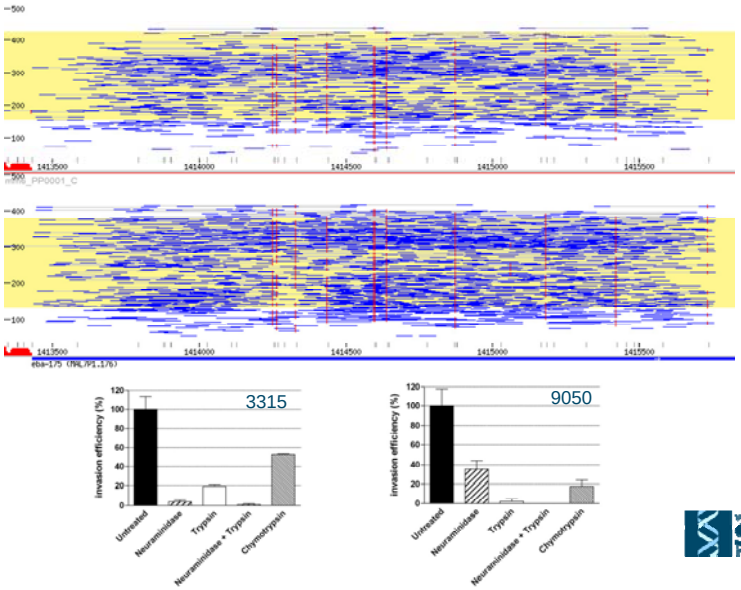
Genotype/phenotype correlations - candidate genes



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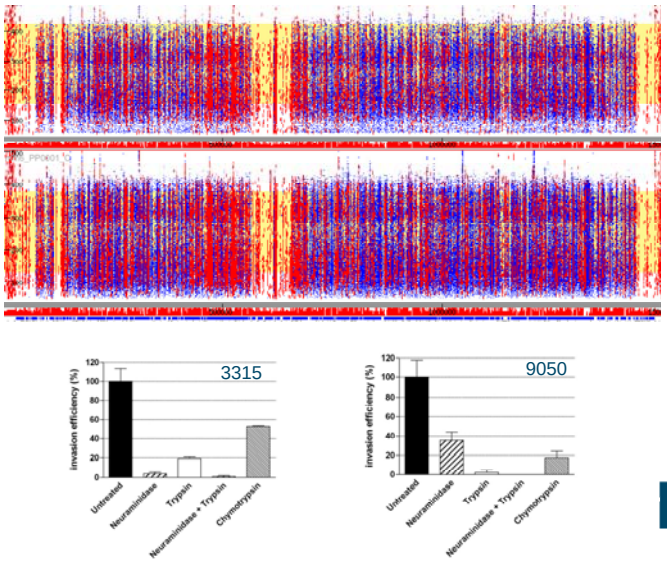
Genotype/phenotype correlations - Illumina c/s 7



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Thanks for your support

Team 115
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Mike Blackman (National Institute for Medical Research/UCL)

Thesis Committee:
Oliver Billker
Matthew Higgins

Funding
The Gates Cambridge Trust
Wellcome Trust

Collaborators/Special Assistance
Katrin Volkmann (Team 131)
Bee Ling Ng (LSR II facility)
Magnus Manske (LookSeq)
Malaria Teams – Billker, Kwiatkowski

Faculty enabling Research Opportunity
Annabel Smith/Christina Hedberg
Julian Rayner
Alex Bateman
Dominic Kwiatkowski
David S. Roos (UPenn)
Ponzy Lu (UPenn/Vagelos program)

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Thanks for your support

Facilitation of Thesis Defense
Invigilator: Brian Sullivan (Washington University in St. Louis)
Board of Graduate Studies – Univ. Of Cambridge
Anthony Phillips (WashU Videoconferencing facility)
WashU Medical Scientist Training MD/PhD Program

Thesis Defense References

Aikawa, M., Miller, L.H., Johnson, J., and Rabbege, J. (1978). Erythrocyte entry by malarial parasites. A moving junction between erythrocyte and parasite. J Cell Biol *77*, 72-82.

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