Development of a High-Throughput Bioassay to Determine the Rate of Antimalarial Drug Action using Fluorescent Vitality Probes

A thesis submitted in fulfilment of the

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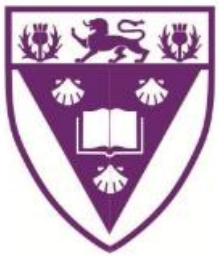

By

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Abstract

Malaria is one of the most prevalent diseases in Africa and the *Plasmodium falciparum* species is widely accepted as the most virulent, with a fatality rate of 15 – 20 % of reported cases of infection. While various treatments have been accepted into early stage clinical trials there has been little progress towards a proven vaccine. Pending a long term solution, endemic countries rely heavily on the development of innovative drugs with acute efficacy coupled with rapids mode of action. Until recently the rate of drug action has been measured by light microscopic examination of parasite morphology using blood slides of drug treated parasite cultures at regular time intervals. This technique is tedious and, most importantly, subject to interpretation with regards to distinguishing between viable and comprised parasite cells, thus making it impossible to objectively quantitate the rate of drug action. This study aimed to develop a series of bioassays using the calcein-acetoxymethyl and propidium iodide vitality probes which would allow the rate of drug action on *Plasmodium falciparum* malaria parasites to be assessed and ranked in relation to each other. A novel bioassay using these fluorescent vitality probes coupled with fluorescence microscopy was developed and optimized and allowed the rate of drug action on malaria parasites to be assessed i) rapidly (in relation to current assay techniques) and ii) in a semi-quantitative manner. Extrapolation to flow cytometry for improved quantification provided favourable rankings of drug killing rates in the pilot study, however, requires further development to increase throughput and approach the ultimate goal of producing a medium-throughput assay for rapidly assessing the rate of action of antimalarial drugs. Attempts to adapt the assay for use in a multiwell plate reader, as well as using ATP measurements as an indication of parasite vitality after drug treatment, was met with erratic results. The viability probes assay as it stands represents an improvement on other assay formats in terms of rapidity and quantification of live/compromised parasites in cultures.

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List of Abbreviations

ACT	Artemisinin-based combination therapy
AM	Acetoxymethyl
AMP	Adenosine monophosphate
APAD	3-Acetylpyridine adenine dinucleotide
APADH	3-Acetylpyridine adenine dinucleotide, reduced
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CM	Complete Medium
DAPI	4,6-diamidino-2-phenylindole
ddH_2O	Double distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
HE	Hydroethidine

HIV	Human Immunodeficiency Virus
iRBC	Infected red blood cell
MB	Methylene Blue
MMV	Medicines for Malaria Venture
MSF	SYBR Green I-based fluorescence
NBT	Nitro tetrazolium blue
PBS	Phosphate-buffered saline
PES	Phenazine ethosulphate
P. falciparum	*Plasmodium falciparum*
PfSUB-1	Plasmodium falciparum subtilisin-like protease 1
PI	Propidium iodide
pLDH	Plasmodial lactate dehydrogenase
PPi	Pyrophosphate
PRR	Parasite Recovery Ratio
RBC	Red blood cell
RFU	Relative fluorescence units
RHX	Radioactive hypoxanthine
RLU	Relative luminescence units
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
TMRE	Tetramethylrhodamine
USA	United States of America
UV	Ultraviolet
WHO	World Health Organisation
WI	Wisconsin

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Chapter 1 – General Introduction

1.1 - Overview

Malaria is one of the most prevalent diseases in Africa and is caused by parasites of the genus Plasmodium. Described as a 'prehsitoric' parasite, its associated symptoms have been documented as early as 2700BC, but it wasn't until 1880 that Charles Louis Alphonse Laveran identified the instrumental agent as a parasite (Cox, 2010). Various *Plasmodium*species have been identified with differential geographic distribution, including *Plasmodium ovale, malariae,* and *vivax*. These species have been shown to have low levels of drug resistance and rarely cause fatalities (Janneck *et al*., 2011). Alternatively, the *falciparum (P. falciparum*) species is widely accepted as the most virulent, with a fatality rate of 15 – 20 % of reported cases of infection (Mace *et al*., 2011). Some of the severe symptoms can include renal failure, pulmonary edema and anemia, alongside its increasing resistance to multiple anti-malarial drugs (Janneck *et al*., 2011). It is often the case that when an individual becomes symptomatic of malaria their health can deteriorate rapidly.

The World Health Organization's (WHO) annual malaria report proposed its primary goal as being a reduction in malaria related deaths to near zero by the end of 2015 (WHO, 2013). Due to their developing immune systems, children under the age 5 have been identified as the most susceptible to the disease (Black *et al.,* 2003). While various treatments have been accepted into early stage clinical trials there has been little progress towards a proven vaccine. Until a long term solution is found endemic countries rely heavily on the development of innovative drugs with acute efficacy coupled with rapid modes of action.

1.2 - Life Cycle

Human transmission (Figure 1) occurs via the female mosquito of the genus *Anopheles* whereby the parasite is transferred to and invades the host red blood cells (RBCs) and develops through ring, trophozoite and schizont life stages (Fu *et al.,* 2010).

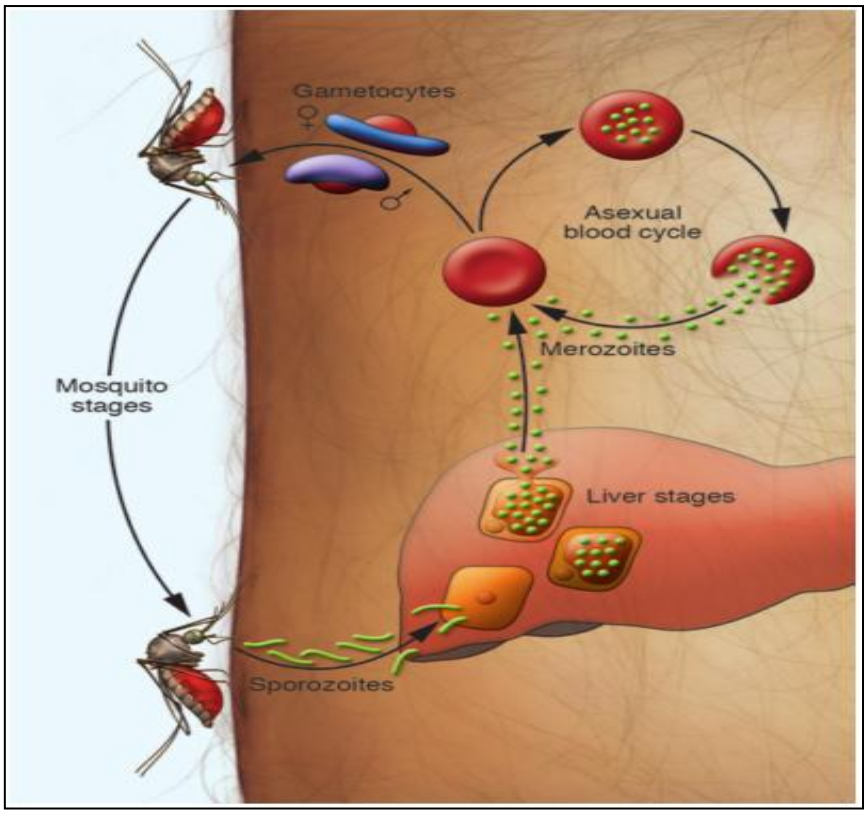

Figure 1: Life cycle of malaria parasite showing both asexual and sexual life stages.The asexual blood stage is the most commonly studied and accounts for the acute parasitic infection and accompanying symptoms (Wellems *et al*., 2009).

The female *Anopheles* mosquito produces eggs throughout her life and requires relatively large amounts of protein for their production. This protein is acquired from the blood they feed on making them a model vector for malaria transmission (Miller *et al.,* 1994). Part of the feeding process involves the mosquito injecting salivary fluid containing anti-coagulants into the blood of the host, and if infected with malaria, allows the transfer of sporozoites into host

tissue or directly into the blood stream (Baldacci and Menard, 2004). The sporozoites then travel to the liver where they infect liver cells and begin dividing asexually into merozoites marking the onset of the asexual life cycle (Grimberg, 2011). Once significant replication has taken place the live cells swell and rupture releasing the merozoites into the bloodsteam where a single merozoite will infect a RBC and initiate the intraerythrocytic stage of the life cycle.

When inside the host RBC the merozoite adopts a flattened morphology where the cellular organelles are found around the periphery of the parasite while the centre contains only a thin layer of cytoplasm (Bannister *et al.,* 2000). Under light microscopy this morphology takes on the appearance of a 'ring', hence the name 'ring-stage parasite'. The ring stage occurs from 0 – 20 hours as the RBC is slowly altered while a strict set of genes responsible for parasite metabolism are transcribed (Fu *et al.,* 2010). The primary mode of parasite metabolism involves glycolysis and digestion of haemoglobin and the subsequent release of haem which is packed into crystals called haemozoin (Grimberg, 2011). The latter occurs during the trophozoite stage (20 – 36 hours) where metabolism is exponentially increased. It has been observed that lactic acid production in a trophozoite stage, parasite infected RBC is increased up to 100-fold in relation to an uninfected RBC (Pfaller *et al.,* 1982).

Once the host RBCs haemoglobin has been fully metabolised, the mature trophozoite initiates nuclear division and subsequently enters the shizont stage where large scale DNA synthesis is observed (Fu *et al.,* 2010). After a period of 36-48 hours and once DNA and organelle replication is complete, new cellular components are organized into independent foci, and after segmentation, produce new daughter merozoites. Each merozoite is then able to infect a new RBC and so maintains the intraerthryocytic stage of the parasite life cycle (Kirk, 2001; Cowman and Crabb, 2006).

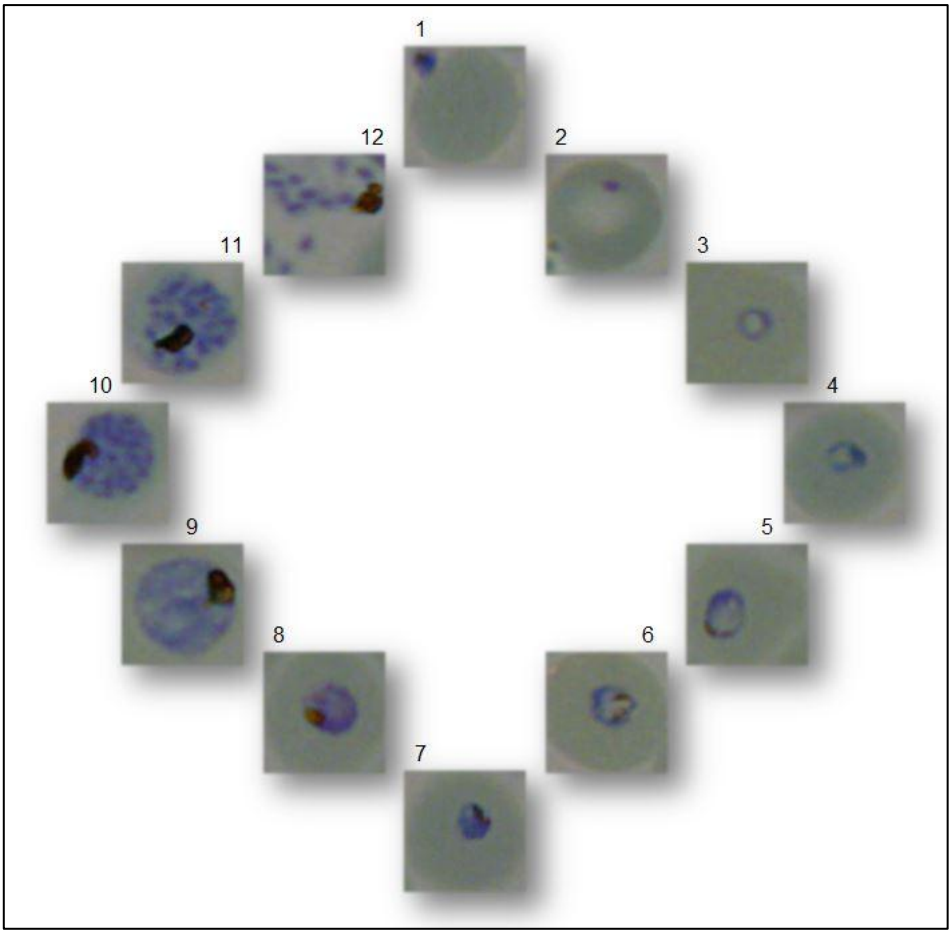

Figure 2: Light microscope images of Giemsa-stained *P. falciparum* parasites depicting the sequential intraerythrocytic life cycle. The cycle is initiated by the invasion of a merozoite into a host RBC (1-2). Once inside, the early stage parasite forms a ring-shaped structure and initiates hemoglobin metabolism (3-5). Once the parasite matures it enters the trophozoite stage and metabolism is exponentially increased (6-9). The parasite then undergoes asexual replication as it enters the shizont phase (10-11) and approximately 48 hours post RBC invasion the host ruptures releasing daughter merozoites into the bloodstream (12).

Alternatively, some trophozoites may differentiate into gametocytes and reinitialize the sexual stage of infection. The signal by which this process occurs is not yet clear although parasite stress has been found to be a contributing factor (Fivelman *et al*., 2007). In the case of *P. falciparum* gametocyte differentiation is observed only a few weeks after erythrocyte infection (Fivelman *et al*., 2007). As gametocytes are incapable of producing male and

female gametes within the human host, they differentiate only when extracted by another mosquito. Once sexual development is complete the newly formed sporozoites are deposited into the salivary glands of the mosquito from where they are transferred to a new human host. (Grimberg, 2011).

1.3 - Geographic distribution and impact

As all malaria species make use of the female *Anopheles* mosquito as a vector it has been shown that malaria endemic regions often significantly overlap with the *Anopheles* mosquitos' idyllic environment (Hay *et al.,* 2009). This relates to the tropic regions of the world (Figure 3) where an estimated 1.4 billion people are at risk of contracting the parasite, excluding all the travellers that visit these regions throughout the year (Hay *et al.,* 2009). Important to note is that developing countries dominate these tropical regions and the high levels of poverty and inadequate healthcare lead to increased problems in controlling transmission.

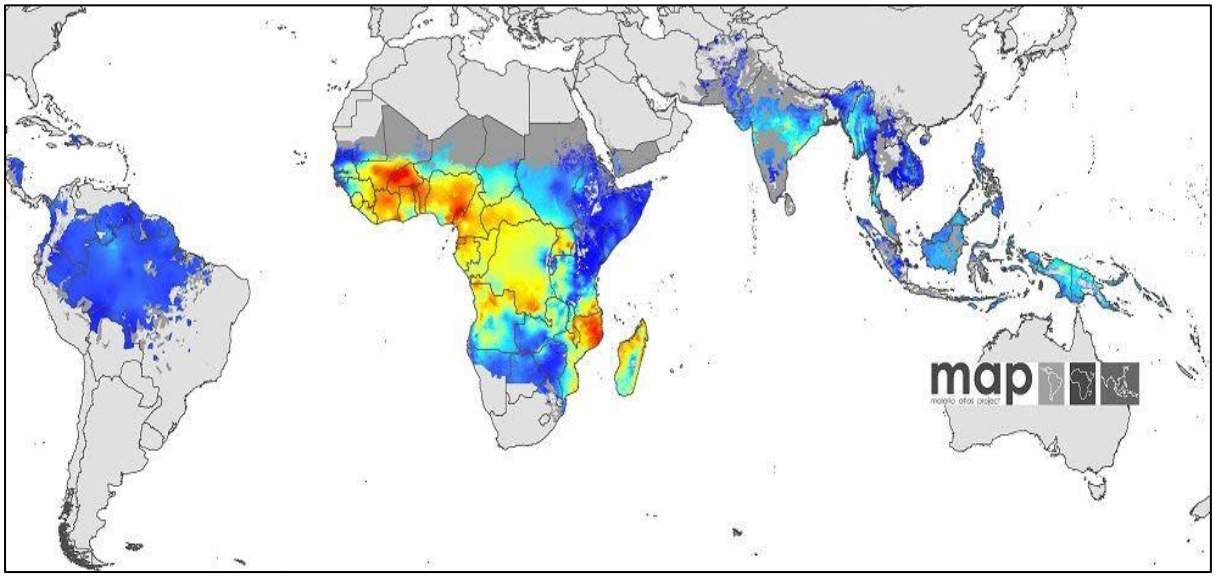

Figure 3: Stable *P. falciparum* malaria transmission regions as of 2010 (where a scale of blue (lowest transmission) to red (highest transmission) is indicated). Of the 1.4 billion people living at risk of malaria, sub-saharan Africa, the South Americas and south-east Asia carry the highest malaria burden.

Africa has the highest risk of infection accounting for 90% of all fatalities (adapted from Gething *et al.*, 2011).

From a South African perspective it has often been the ideology that preventative measures need only be taken when travelling to central Africa. This is true in the sense that the regions with the highest prevalence of malaria are along the border of Mozambique. However, this notion is rapidly becoming obsolete as Tonnang and company showed that due to climate change distribution patterns of the *Anopheles* mosquito have been shown to be expanding to the east and south-east coasts of South Africa (Tonnang *et al.*, 2010). South Africa is fortunate in relation to other developing countries in Africa and Asia in the sense that it has relatively well developed health care infrastructure and is economically stronger, therefore being able to better implement malaria control measures.

1.4 - Current malaria vector control

Development of innovative means of vector control has been slow. There are essentially three broad ways of tackling the issue. The first is a prophylactic approach in order to prevent the initial acquisition of the parasite. This involves the use of insecticide treated bed nets and indoor residual spraying. Although these options may seem outdated, they are still the most effective ways of preventing infection (Ridley, 2001). This approach is of concern, however, with the emergence of insecticide resistant mosquito vectors and therefore requires constant monitoring as well as novel, environmentally friendly insecticide and insecticide-combination treatments (Klausner and Alonso, 2004). Recently there are environmental management programs aimed at reducing mosquito breeding sites (Klausner and Alonso, 2004).

The other alternative prior to acquisition of the disease is the use of a vaccine. A target specific vaccine has yet to leave developmental stages, therefore this option is unavailable in malaria treatment. Only a single vaccine has progressed as far as stage 3 clinical trials -

namely RTS,S/AS01 (Agnanji *et al.,* 2012). Its results have been somewhat disappointing with minor decreases in the risk of infection in babies vaccinated between 6 and 12 weeks of age (Agnanji *et al.,* 2012). Other vaccines do exist but are at least 5-10 years behind RTS,S/AS01.

Acquisition of the disease leads to the final method of control – drug treatment. The key to malaria drug treatment is early diagnosis and rapid action as it is an acute infection (as opposed to a chronic infection like HIV). If the disease is left unchecked and proceeds to the severe form, hospitalization is required. As malaria is extremely prevalent in poor and developing countries with severe shortages in adequate health care, this provides a major challenge to current control measures. Poor economic development leads to limited access to drugs and trained staff (Ridley, 2001). With reports of increasing vector resistance to insecticides and the lack of a vaccine, drug treatment remains the last line of defence against malaria (Grimberg and Mehlotra, 2011).

1.5 - Drug Treatment

Since synthetic chemicals were introduced for malaria control over 70 years ago only a small number of drugs (belonging to three classes) have reached the market and with increasing reports of resistance there is a drive to alter and combine the existing anti-malarials to create better drug formulations (Ridley, 2001).

1.5.1 - Quinolines

Quinine was originally extracted from cinchona bark in the 1800s and is often the go-to drug for severe forms of malaria (Grimberg and Mehlotra, 2011). Along with its quinoline derivatives it was used to treat the symptoms of malaria before the parasite was even identified as the causative agent (Meshnick and Dobson, 2001). Chloroquine (a derivative of

quinine) has been the most widely used anti-malarial due to its high rate of success and low cost. Mechanisticallyit is widely accepted that it accumulates in the parasite food vacuole where the digestion of haem takes place (Chinappi *et al.,* 2010). Proteases exist within the food vacuole which digest host RBC haemoglobin releasing haem, a toxic moiety to malaria parasites (Yazon *et al.,* 1984). The parasite then sequesters the haem into a haemozoin crystal resulting in haem detoxification. Chloroquine is proposed to disrupt the sequestration process resulting in a build-up of toxic haem and resultant cell death (Chinappi *et al.,* 2010).

Unfortunately, it has become increasingly redundant due to widespread resistant strains covering a vast majority of malaria endemic areas (Figure 4).

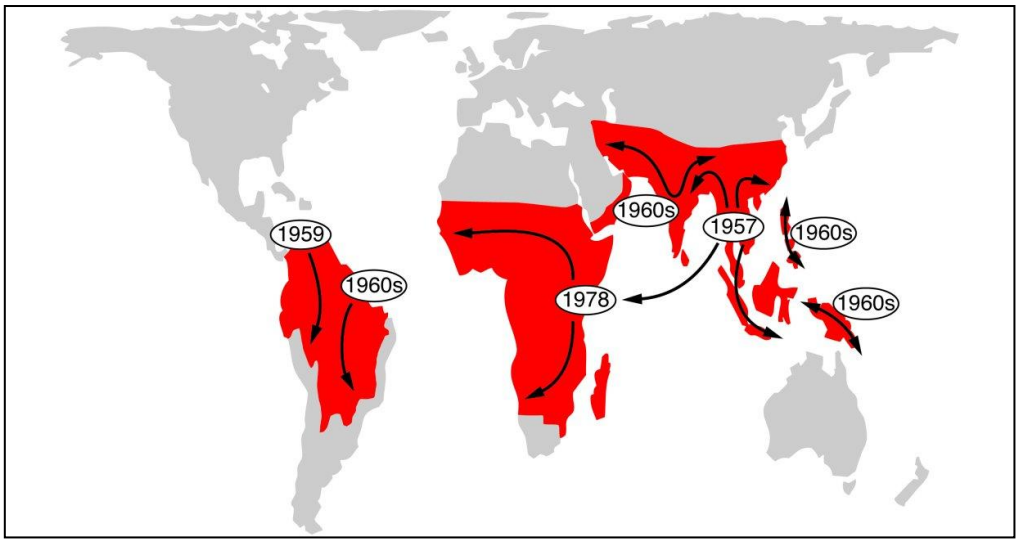

Figure 4: Global regions with most widespread chloroquine resistance (indicated in red) in *Plasmodium falciparum.* Initial resistance is shown to have originated in South-East Asia and spread rapidly (indicated by arrows) across Africa and South America. (Wellems *et al.,* 2009).

As with its mechanism of action, the mode by which resistant parasites deal with an influx of chloroquine has been hypothesized by a variety of studies. Krogstad and associates (1988) suggested a mechanism whereby resistant *P. falciparum* parasites have adapted a process for rapid efflux of chloroquine (Krogstad *et al.,* 1988). This efflux is then absent or significantly

reduced in wild type malaria. Variousmechanismshave been proposed and include a reduction in the alkalinity of the chloroquine molecules such that it is no longer able to accumulate inside the food vacuole (Krogstad *et al.,* 1987); the development of a permeability barrier around the food vacuole disallowing chloroquine sequestration (Hancock, 1987); or an enzyme which perhaps inactivates chloroquine (analgous to β-lactamases) (Neu, 1987). However, it is now widely accepted that chloroquine resistant strains of malaria are caused by mutations on two genes associated with the parasite food vacuole – a chloroquine resistance transporter (*Pfcrt*) and the multidrug resistance transporter-1 (*Pfmdr1*), with the *Pfcrt* mutation being a stronger predictor of resistance than the *Pfmdr1* mutation (Mohammed *et al.,* 2013). Both *in vitro* and clinical resistance has been observed with the specific *Pfcrt*-K76T mutation and it has been shown that the removal of this mutation from resistant strains led to wild-type levels of chloroquine sensitivity (Lakshmanan *et al.,* 2005)

Mefloquine is a quinoline methanol derivative used as both a therapeutic and prophylactic antimalarial. Due to its more potent activity, in relation to chloroquine, it was quickly marketed under the name Lariam™ by Hoffman La Roche for U.S. military deployment to malaria endemic regions. In contrast to chloroquine, it had a longer half-life allowing for a single weekly dose thereby reducing the risk of non-compliance. Sadly due to its chemical makeup being so similar to that of chloroquine, resistance emerged quickly and limited its accessibility to developing countries (Nosten *et al.,* 1994). There is evidence to suggest a similar mechanism to that of chloroquine such that, like chloroquine, mefloquine may interfere with parasite haemoglobin metabolism preventing growth and resulting in cell death. This has been proposed to occur either by mefloquine binding directly to haem (Dorn *et al.,* 1998), degrading the haem group (Loria *et al.,* 1999) or inhibiting the polymerization of haem to haemozoin (Sullivan *et al.,* 1998). Alternately there is also substantiation of additional or different modes of action to that of chloroquine as mefloquine has been shown

to exhibit higher degrees of activity against chloroquine resistant strains of malaria (Mungthin *et al.*, 1998; Zhang *et al.*, 1999). A study that focused on the effects of mefloquine and chloroquine on parasite endocytosis proposed that mefloquine acts on early stage haemoglobin metabolism by inhibiting host cell cytoplasm ingestion while chloroquine appeared to act later on by blocking the digestion of haemoglobin (Hoppe *et al.*, 2004).

1.5.2 - Antifolates

In an effort to provide more adept means of combating leukaemia, antifolate agents were developed. This solution was short lived as it was found not only to be ineffective but in some cases to cause an increase in disease malignancy (Farber *et al.*, 1948). It did however show that folic acid was an important factor in the spread of leukaemia and thus led to the first folate analogue, aminopterin which demonstrated a drastic increase in remissions in cancer patients and a landmark for cancer chemotherapy (Farber *et al.*, 1948). This success led to the use of this class of drugs in the treatment of other rapidly dividing cells such as parasites.

The antifolate class of drugs used to counter *P. falciparum* can be divided into two groups – those which target the parasitic dihydropteroate synthase (class I antifolates) and those which target the dihydrofolate reductase (class II antifolates) (Nzila, 2006). Arguably the best recognised of the antifolates is pyrimethamine which belongs to class II and as such counters the parasitic dihydrofolate reductase and results in reduced ability for DNA and amino acid synthesis (Hurwitz *et al*., 1981). Used less in monotherapy, it is often used in combination with sulfadoxine and sulfalene but is being prescribed less frequently as within a decade of its introduction widespread resistance (Figure 5) was encountered in South America and South-East Asia (Hurwitz *et al*., 1981).

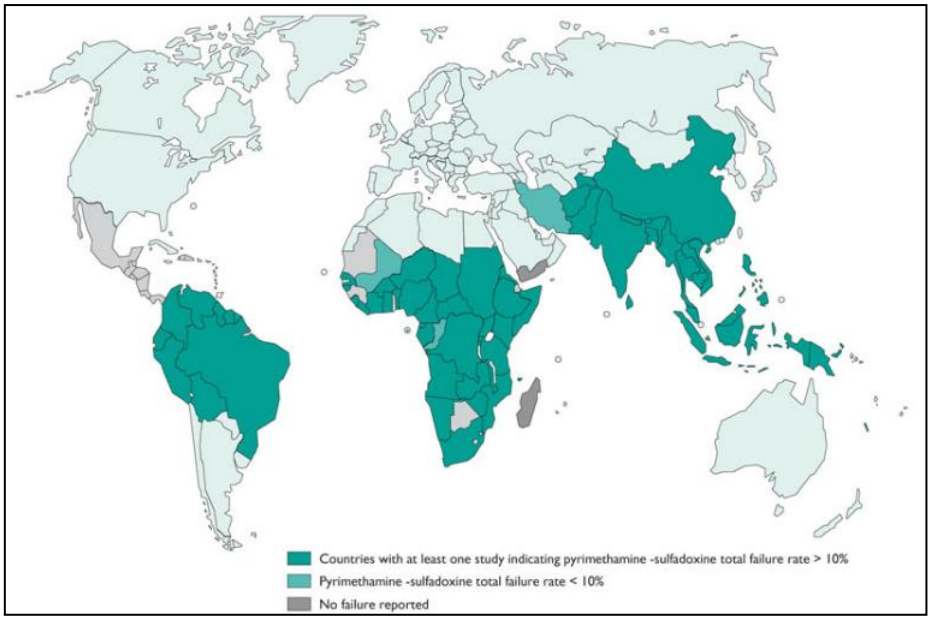

Figure 5: Distribution of sulfadoxine-pyremethamine resistance in *Plasmodium falciparum.* Regions affected correlate with those mentioned to show resistance to quinoline derived drugs (Anderson, 2003).

1.5.3 - Artemisinins

The current global standard for malaria treatment is the use of artemisinin and its derivatives. Like the quinine class, artemisinin is also derived from plants, the qinqghao herb, and was initially used as a treatment for fevers in traditional Chinese medicine (Klayman, 1985). In 1977 the structure was solved by Qinghaosu et al., and provided the basis of a chemical scaffold which could be modified to improve solubility and synergy with other antimalarial drugs (Klayman, 1985). To date artemesinin is still extracted from the qinghao and with its derivatives comprises the last and most effective line of defence against modern, resistant malaria strains. Reasons for its versatility includes the fact that its administration can take one of several routes – intramuscular, intravenous, intrarectal and oral (Hien, 1994; Krishna *et al.*, 2004). A relatively short half-life is observed in comparison to other common antimalarials – the active metabolite has an elimination half-life of approximately 1 hour (Krishna *et al.*,

2004) and it has shown to be effective against severe strains of malaria that are highly resistant to chloroquine (Agbenyega *et al.,* 2000). Artemisinins have been shown to impede the broadest range of malaria parasite life stages whereby effect has been shown to be pertinent in rings, trophozoites and early stage shizonts (ter Kuile *et al.*, 1993; Angus *et al.*, 1997).

Due to the emergence of resistance to previous antimalarials, over an arguably short time, the WHO has recommended the use of artemisinin combination based therapies (ACT). ACT involves making use of artemisinin or several related endoperoxide antimalarials which rapidly eliminate *P. falciparum* infections in combination with drugs with longer half-lives and spanning different classese.g. artesunate-mefloquine (Dondorp *et al*., 2010). This is done to combat any remaining parasites after initial artemisinin action. Although widespread resistance of artemisinin and its derivatives have begun to show reduced susceptibility of *P. falciparum* in the Cambodia-Thailand region (Figure 6) and neighbouring areas in south-east Asia (World Malaria Day, 2012). Chloroquine and pyremethamine resistance can be traced back to the same origin – Cambodia, where in both cases the resistant strains swept across Africa and killed millions of people (White, 2010). The alarming reality is that artemisinin resistance has become apparent in Cambodia and neighbouring countries in Southeast Asia and if resistance is allowed to spread as per previous drugs the results will be catastrophic. Renewed funding has prompted malaria drug research after nearly 10 years of absence but the drug development process is slow. Currently the only compounds in the pipeline to replace ACTs are other ACTs and whether they will kill multi-resistant strains is unknown (Enserink, 2010).

Figure 6: Artemisinin resistance present in Southeast Asia originating in Cambodia and show rapid spread to Myanmar, China and Vietnam (World Malaria Day, 2012).

1.6 - Antimalarial Drug Discovery

Antimalarial drug discovery is an extremely challenging process due to the environment in which they are most needed. This is based on the fact that malaria endemic areas are some of the poorest in the world and access to suitable health care is limited or non-existent. Any novel antimalarial compound has to be well tolerated by the patient and have minimal side effects. This leads to the necessity for the drug to be readily available for oral administration in a rural setting. One of the primary reasons for drug resistance is the lack of patient compliance over the full period of therapy. It is therefore necessary to provide a treatment that will act quickly and last approximately three days with as few dosings as possible. The drugs should also be used in conjunction n with other drugs (as is the case with artemisinin) to reduce the risk of resistance. Finally the drugs should be inexpensive (less than $ 0.1, as is the case with chloroquine) (Gelb, 2007).

The extreme difficulty which exists when attempting to culture and isolate gametocytes makes drug development directed towards the sexual stage of the parasite almost nonexistent. This is in contrast to the relative ease it takes to generate asexual parasite culture. The first straight-forward protocol to be published for *P. falciparum* culture was by Trager and Jensen (1976) and has since been optimized and refined to become a standardized protocol. Some of these advancements include the use of AlbuMax II (a synthetic serum) instead of the more costly human sera, as well as using a specialized gaseous atmosphere (1 % O_2, 94 % N_2 and 5% CO_2) for which more successful growth has been shown (McNamara *et al*., 2006).

As it could take many years before a novel antimalarial is successful in drug trials there are currently a variety of alternative measures being used to slow the increase of virulent, drug resistant malaria strains. It has been shown that the removal of a drug from which parasites have become resistant (such as chloroquine) from administration is able to bring back drug-sensitive strains and eventually replace drug-resistant strains in the majority of the parasite population, however, sensitivity to the respective drugs may require up to 10 years to return (Kublin *et al.,* 2003). Alternatively, drugs presently used to treat other diseases can be repurposed to treat malaria if they show reputable activity. This has been shown to be effective with antiretrovirals used commonly to treat HIV as well as anticancer drugs such as SU-11274 and Bay 43-9006. These drugs, however, bring with them additional complications in terms of toxicity, cost and efficacy (Grimberg *et al.,* 2009).

Additional alternatives include synthetically altering existing compounds in in order to preserve drug efficacy while simultaneously reducing or avoiding pre-existing resistance (Vangapandu *et al.,* 2007) which has been shown to be effective in mefloquine and chloroquine. There is also the option of drug hybridisation whereby two closely related or class distinct compounds are covalently linked to circumvent the issue of singular drug

resistance. Previous work has shown this to be effective in artemisinin-quinine based hybrids in which strong activity was shown against chloroquine-resistant strains of *P. falciparum* (Walsh *et al.,* 2007). Marine and plant natural compounds are increasingly sought after due to their historical medicinal properties but this is an exceptionally lengthy process including screening procedures, species specificity and selection, as well as isolation experimentation (Ginsburg *et al.,* 2011). Target-based drug discovery has become a topic of interest since the sequencing of the parasite and human genomes and drug targets have already been identified, including kinases and falcipains to name a few (Grimberg and Mehlotra, 2011).

A more recent approach has been the development of high-throughput screening techniques using large databases of compounds with antimalarial activity *in vitro* can be assessed for their degree of efficacy and cytotoxicity.

1.7 - *In vitro* assessment of malaria drug efficacy

With an increase in *in vitro* efficiency of malaria culture comesa cumulative need for the development of adept ways of studying parasite sensitivity to antimalarial compounds. The initial and still most widely used technique is the use of the Giemsa stain in combination with microscopic examination of blood smears for parasite diagnosis (Fleischer, 2004). Although this technique has provided the majority of data on parasite vitality in culture, it is prone to misdiagnosis and subjective interpretation (Grimberg, 2011).

The use of radioactive hypoxanthine (RHX) attempted to improve on the use of a microscope as the RHX would be incorporated into the parasite DNA as it was synthesized (Desjardins *et al*., 1979). Erythrocytes don't synthesize polynucleotides, hence RHX incorporation is a specific indicator of parasite levels in cultures. Although an improvement over microscopy, RHX is not without its downfalls i.e. the need for a radioactive H^3-purine and specialised instrumentation (Desjardins *et al*., 1979). An alternative detection method was parasite

enzyme specificity for e.g. the plasmodial lactate dehydrogenase (pLDH) assay. This assay is based on the difference noted in *P. falciparum* which rapidly uses 3-acetyl pyridine in the reaction to produce pyruvate from lactate, in comparison in humans the same reaction occurs 200 times slower (Makler and Hinrichs, 1993). The assay selectively detects parasites pLDH vs. host RBC's LDH in culture. The pLDH assay has become the assay of choice over the RHX assay due to its simplicity and relative inexpense in comparison.

ATP quantification in mammalian cells can be measured using isolated mitochondria and fluorometry (Tatuch and Robinson, 1993; Houstek *et al.*, 1995; Wanders *et al.*, 1996), however, an economical and robust way to measure ATP in both live cells and isolated mitochondria makes use of the luciferin-luciferase reaction whereby luciferin luminescence is measured after being catalysed by firefly luciferase (Lyman and DeVincenzo, 1967; Manfredi *et al.*, 2002):

$$Luciferase + luciferin + ATP + O_2 \longrightarrow luciferase + oxyluciferin + AMP + PPi + CO_2 + light.$$

This reaction is exploited in commercially available kits (e.g. Promega's Cell-Titer-Glo and BacTiter-Glo) to quantify ATP levels in mammalian and bacterial cell cultures as a measure of cell viability.

Previous studies have indicated that the luciferin-luciferase reaction is effective in measuring ATP levels in asexual and sexual stages of *P. falciparum* parasites (Khanaani and Ginsburg, 1989; Peatey *et al.*, 2012). Recent developments in the field have seen Khan *et al.*, (2012) explore the possibilities of utilizing native parasite ATP levels as an indication of drug stress and if the quantification of ATP levels would be suggestive of the rate of antimalarial drug action. The study observed an increase in ATP levels in the parasite post drug treatment, suggesting that the harmful effects of the drugs triggered an increase in metabolic activity and

subsequent elevated ATP levels in response. In converse, decreased ATP levels would relate to a decline in parasite metabolism and viability (Khan *et al.,* 2012).

Fluorescence microscopy-based drug-screening procedures have been developed and make use of the concept that host RBC's lack DNA and hence parasite growth can be measured using nucleic acid stains such as SYBR green and DAPI. The SYBR Green I-based fluorescence (MSF) assay has been used to monitor drug efficacy and associated resistance and involves lysing infected RBCs in culture post drug-treatment using saponin (a nonlethal detergent that removes the parasite from the host RBC) after which the SYBR green fluorescent probe is added to the culture sample and relative fluorescence units (RFU) are calculated per sample in a multiwell plate reader (Smilkstein *et al.,* 2004; Johnson *et al.,* 2007). The MSF assay allows for a reliable, high-throughput, automated, plate based method to measure malaria susceptibility to current and novel antimalarial drugs (Johnson *et al.,* 2007). Linked to flow cytometry, these assays can be used to provide quantitative screens based on an exact parasite cell count in patient samples as well as cultures. In addition, a fluorimeter may be used to measure parasite-associated fluorescence in multiwell plates, providing a convenient means for assessing the pertinent issue of drug efficacy against cultured malaria parasites (Moneriz *et al.,* 2009).

Although these assays are an improvement on conventional microscopy, there are disadvantages. Compounds are typically added to cultures in 96-well plates and residual parasite levels determined using the assays after a 48-96 hour period. They are not stage specific and there is no way of accurately determining the rate of drug action on the parasite. This factor has become increasingly crucial to understand especially due to factors such as poor health care and patient compliance in malaria endemic regions. Rapid-action drugs also provide opportunities to combat the severe forms of malaria where prompt action is crucial to

prevent death in patients. Current methods for determining rate-of-death in malaria parasites hinge on using light microscopy with Giemsa stained blood smears of parasite cultures to examine the deterioration in parasite morphology during drug exposure. This technique is labour intensive, time consuming and prone to subjective interpretation i.e.determining viable cells from non-viable. It is impossible to achieve an accurate quantification of parasite viability. Therefore new techniques need to be developed to quantitatively assess the rate of antimalarial compounds and drug action in parasite culture. One such approach is to use fluorescent vitality probes to definitively distinguish between viable and compromised populations of drug treated parasites.

1.8 – Fluorescent cell staining

Innovation in the fields of molecular and cellular biology has provided a plethora of methods to study individual structures and whole cell functions thereby allowing the use of fluorescent compounds to selectively target and visualize various cellular activities and structures i.e. cellular membranes, proteins and nucleotides. Some fluorescent compounds are able to distinguish between viable and non-viable cells and are known as vitality probes. All membranes have an electrical potential difference which exists across them generating a negative environment inside the cell, therefore fluorescent cell staining functions on the basis that positively charged molecules can readily pass through viable cell membranes and, depending on downstream hydrolysis, will remain inside the cell. Negatively charged compounds are unable to pass through viable cell membranes and hence are often used as ‘dead’ or compromised-cell indicators.

There are primarily three modes of vitality probe staining – nuclear, mitochondrial and cytoplasmic. Cytoplasmic staining involves engineering fluorescent compounds such as calcein and hydroethidine into neutral molecules by the addition of an acetoxymethyl group.

The neutral probe can freely permeate cell membranes and subsequently be converted into their fluorescent products via cytoplasmic esterases (Figure 7A and 7C). These compounds are then retained by the cell due to their ensuing negative charge. Mitochondrial membrane potential has a significant impact on the integrity and activity of these organelles and can be altered by a variety of intra- and extra-cellular signals. As such dyes have been developed, such as tetramethylrhodamine (TMRE), to act as indicators of mitochondrial membrane potential and ultimately cellular stress (Lyons and Parish, 1994). These probes are often positively charged and readily cross cellular and mitochondrial membranes where they are retained in healthy mitochondrial matrices (Figure 7B). An accumulation of probe hence causes an increase in fluorescence. Nuclear staining involves fluorescent probes with aromatic amino groups such as propidium iodide (PI) which interact with DNA and RNA directly to produce fluorescence. PI molecules intercalate within the double helix while probes like 4',6-diamidino-2-phenylindole(DAPI) interact with the minor groove of the DNA. These probes are negatively charged and cannot readily permeate cellular membranes. A loss of membrane integrity is the foremost indicator of cellular death and hence these probes can be used to detect cellular degradation (Figure 7D).

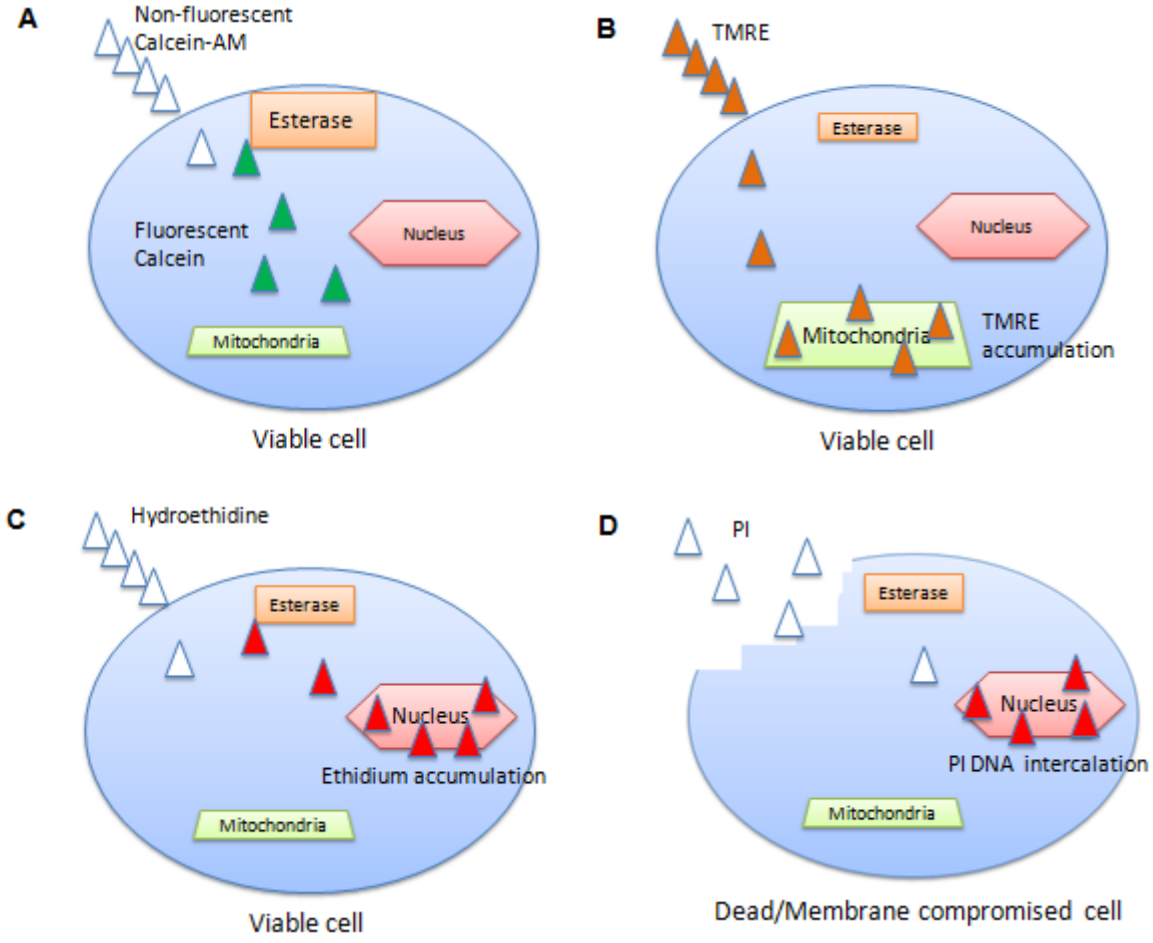

Figure 7: Diagrammatic representation of fluorescent vitality probes and their area of intracellular sequestration. A – Calcein-AM freely permeates cellular membranes and is cleaved by intracelluar esterases to green fluorescent calcein. B – TMRE readily permeates cellular membranes and is retained by healthy mitochondria exhibiting orange fluorescence. C – Hydroethidine freely permeates cellular membranes and is cleaved to ethidium which subsequently interacalates with DNA and produces red fluorescence. D – Propidium iodide is only able to permeate membrane compromised cells where it binds directly to DNA nucleotides and produces red fluorescence.

1.8.1 - Tetramethylrhodamine

Mitochondrial membrane potential is required for maintaining functions like Ca^{2+} uptake. Spontaneous depolarization and subsequent loss of this potential was first studied using TMRE (Loew *et al.*, 1993). TMRE is able to cross the membranes of viable mitochondria and

due to this potential is retained in the cytosol of the mitochondria. As such a rapid increase of orange fluorescence can be detected while a loss of fluorescence can be attributed to unhealthy/dead mitochondria and hence provides an indication of oxidative stress (O`Reilly *et al.*,2003). TMRE has been used very rarely for applications other than mammalian cell staining. In studies that it has been employed for malarial cells it has been used as a parasite screeningprobe to detect the presence of the parasite (Enderle *et al.,* 1997). Similarly it was used to develop a fluorescence-based assay for use in screening for inhibitors of a malarial protease PfSUB-1 whereby TMRE was used to assess the parasites morphological response to said inhibitors with a decrease in fluorescence attributed to an effective anti-PfSUB-1 response (Blackman *et al.,* 2002).

1.8.2 - Hydroethidine

Hydroethidine (alternatively known as dihydroethidium) is a common superoxide detector and is synthesizedvia the reduction of an ethidium cation (Zielonka *et al.,* 2005). As such it has been mostly used in studies requiring an intracellular superoxide probe that does not contribute to the generation of additional superoxide species (Zielonka *et al.,* 2005). A neutral molecule that freely permeates cells, the reaction between hydroethidine and superoxide yields a strong red fluorescence. The cationic ethidium product then becomes retained by the cell due to its charge and intercalation with DNA. Its natural cell permeability and retention by viable cells has led hydroethidine to be popular as a vitality probe. After incubation of live human melanoma and murine fibrosarcoma cells with hydroethidine it was shown that an oxidised form of hydroethidine can be visualized as blue fluorescence in the cytoplasm, as well as red fluorescence in the nucleus (Bucana *et al.,* 1986). It was observed that accumulation of the dye depended largely on the metabolic state of the cell, with

compromised cells exhibiting a significantly reduced amount of fluorescence (Bucana *et al.*, 1986).

Use of hydroethidine in parasites was first completed in *Babesia bovis (B. Bovis)* whereby an assay was developed to measure growth and viability of intraerythrocytic protozoa. The red fluorescence generated by ethidium-DNA intercalation allowed for the use of flow cytometry to distinguish infected, viable parasites from uninfected erythrocytes and dead parasites. It was shown to be effective in observing the effect of parasite specific drugs and the subsequent decline in parasite viability (Wyatt *et al.*, 1991). It was later applied to several plasmodium species including *P. berghei* and *P. yoelii* (Cruz *et al.*, 2012) and *P. falciparum* (Elloso *et al*., 1994). It has since been adopted as a reliable viability stain in malaria parasites and has been compared and contrasted to traditional viability probes such as Thiazole orange (Engelbrecht and Coetzer, 2012), Hoechst 33258 (Brown *et al.*, 1980), Hoechst 33342 (Jacobberger *et al.*, 1992), and PI (Krishan, 1975).

1.8.3 – Calcein-acetoxymethyl ester

The acetoxymethyl ester of calcein (calcein-AM) is a non-fluorescent hydrophobic compound that diffuses across the membranes of cells (Tenopoulou *et al*., 2007). Hydrolysis occurs in the cytoplasm via esterases to produce calcein, a strongly green fluorescent alcohol which is well retained by the cell. It is unable to permeate cellular or organelle membranes once cleaved and hence appears as a cytosolic fluorescence. The exception is when calcein-AM is introduced into cellular suspension at a significantly high concentration such that it over saturates intracellular esterases allowing enough time to diffuse across organelle membranes. Due to its brightness and effective retention, calcein-AM has begun to replace more conventional probes such as fluorescein. The calcein-AM assay has previously been established as a technique to measure the cellular ‘labile iron pool’ in the cytosol (Cabantchik

et al., 1996; Kakhlon *et al*., 2002; Esposito *et al*., 2002; Glicktein *et al*., 2005) and can be diversified to work as a mammalian cell viability assay in measuring multidrug resistance in cancerous mouse embryonic fibroblasts (Lin *et al*., 2002) and as a prokaryotic bacterial stain used in the observation of biofilm permeabilization effects (Carlson *et al*., 2008). It has been shown to measure cell viability using flow cytometry in mammalian natural killer cells (Jang *et al*., 2012), plant pathogenic bacteria (Chitarra *et al.,* 2006) and parasite species such as *Plasmodium berghei* (Kenthirapalan *et al.,* 2012). Although it has been primarily marketed as a mammalian cell stain, with optimization it has been used to measure changes in parasite morphology using fluorescence microscopy in *P. falciparum* (Esposito *et al*., 2010), as well as a general parasite detection probe (Wissing *et al.,* 2002; Gelhaus *et al*., 2008).

1.8.4 - Propidium Iodide

A red fluorescent molecule and well known nucleotide intercalator, PI has been used extensively in mammalian cells (Stewart and Stewart, 1994; O'Brien and Bolton, 1995), bacteria (Vesey *et al.,* 1994), and protozoa (Mossallam 2010). It has also been widely used in malarial parasites where it was utilized as an indicator in the isolationof *P. falciparum* by flow cytometry (Boissiere *et al.,* 2012), to detect changes in parasite morphologyvia the antiplasmodial activity of plant steroids derived from *Solanum nudum*(Lopez *et al.,* 2010), as well as an indicator of general parasite cell death (Hemmer *et al.,* 2005). PI is commonly used to detect the presence of dead cells due to its membrane impermeability and hence exclusion from viable cells. Once inside a compromised cell it binds to DNA by intercalating between the bases at a ratio of one dye molecule per 4-5 base pairs of DNA (Suzuki *et al.,* 1997). It is capable of staining both DNA and RNA and once bound fluorescence intensity is magnified at least 20 fold.

As PI is only capable of staining the nuclei of dead or compromised cells and calcein-AM is a viable cell stain they have both previously been used in conjunction as a dual stain assay. This was in an effort to measure the physiological status of the biphenyl degrading bacterium *C.testeroni* (Hiraoka and Kimbara, 2002). It is unclear whether this technique using a combination of PI and calcein-AM has been used in *P. falciparum* or parasites at all.

1.9 - Extrapolation to a quantitative rate-of-action bioassay

The fluorescent probes described above have worked well in mammalian cells for a variety of research based methodologies but has been used to a lesser degree in parasites owing to the need to adapt their staining protocols for parasite use. They have only been used to detect viable parasites following exposure to experimental compounds, i.e. in a time-independent manner. Therefore to expand on improving the screening procedure for novel antimalarials using fluorescent vitality probes and microscopy, one can add the additional variable of time to the assay. This would allow for screenings to not only include an indication of parasite sensitivity to the compound/drug in question, but will provide an indication of the rate of drug action.

Artemisinin, its derivatives, and the combination therapies in which it is used, have had large scale success and this is predominantly due to its rapid action in impeding parasite viability (Balint, 2001). Victims of the acute form of the disease, especially in third world countries, rely greatly on drugs with a rapid onset of action, where the use of compounds with longer acting half-lives can lead to the onset of severe symptoms and death. The additional complication of patient compliance through the full course of drug treatment only further enhances the need for a single dose, rapid acting antimalarial drug. This points to a requirementto accurately and quantitatively determine the rate of drug action on malarial

parasites with a priority to match or exceed the rapid onset of action of artemisinin and current ACT's.

Until recently the rate of drug action has been measured using Giemsa-stained blood slides of drug treated parasite cultures at regular time intervals. This technique is tedious and subject to interpretation with regards to distinguishing between viable and comprised parasite cells. It is increasingly difficult when one is unable to distinguish between compromised parasites as a result of drug treatment or simplyvariations in morphology commonly found under routine culture conditions. It is therefore impossible to quantitatively determine the rate of drug action using this method. A breakthrough in the field came in the form of a study led by Sanz *et al.*2012) in which *in vitro* killing rates of malaria parasites were successfully measured and allowed for a discrimination between different antimalarial modes of action (Sanz *et al.*, 2012). The concept is based on making use of a serial dilution of drug-treated parasites and measuring re-growth patterns over a six day period. This technique, although effective and essentially definitive, is time consuming and limited to a small quantity of drugs able to be processed at a time. This study proposes the development of a new bioassay in which the rate of drug action on malaria parasites can be assessed over a shorter period of time, in a quantitative manner and in a higher-throughput fashion using fluorescent vitality probes.

One means for quantifying fluorescent probe levels in cells is by flow cytometry. The vast majority of flow cytometry used to assess malaria parasite growth and development has been conducted with the use of nucleic acid stains (Karl *et al.*, 2009). Currently, all cytometry investigations on malaria parasites have been based on the fact that uninfected RBCs lack DNA (Grimberg, 2011). Therefore diagnostic tests can be performed by comparing the ratio of RBCs that stain positive for DNA compared to the total number of RBCs and hence an accurate parasitaemia can be achieved. Previous studies by Karl *et al* (2009) show this to be

an efficient technique by making use of the nucleic acid dye SYBR Green to assay for parasitized RBCs over uninfected cells. Furthermore they made the comparison of cytometry in measuring parasite drug sensitivity to conventional methods i.e. the hypoxanthine incorporation assay and light microscopy with Giemsa staining and concluded that cytometry not only provided a more quantitative result but was also less labour intensive (Karl *et al*., 2009). To extrapolate from the use of nucleic acid stains and flow cytometry to enumerate parasites in culture following drug exposure, this study proposes to use flow cytometry to quantify the time-dependent accumulation of fluorescent vitality probes in cultured parasites as a means for rapidly determining the rate of action of antimalarial compounds. To achieve this, the following experimental aims were pursued:

1. Adapt four well characterized fluorescent vitality probes predominantly marketed for mammalian cell staining for use in *P. falciparum* 3D7 strain parasites.
2. Obtain qualitative confirmation via fluorescence microscopy that the optimised vitality probe staining method can be used to determine the rate of action of a panel of standard antimalarial drugs.
3. Perform the assay in a time dependent manner such that the change in the staining pattern of the parasites can be measured in intervals during drug treatmentand the rate of individual drug action can be quantified.
4. Develop a rapid quantitative method for assessing the rate of action of antimalarial drugs using the vitality probesin combinationwith flow cytometry.
5. Explore additional assay techniques with which to measure the rate-of-drug action by using a fluorimeter to quantify vitality probe levels in drug treated parasites.
6. Compare the assays to current methods of determining the rate of drug action i.e. the PRR assay developed by Sanz *et al.* (2012) and the ATP assay developed by Khan *et al.* (2012)

Chapter 2 - Development and Optimization of a *Plasmodium falciparum* Fluorescent Probe Assay to Measure Rate of Drug Action

2.1 - Introduction

One of the principal reasons for drug resistance and treatment failure is the lack of patient compliance over the full period of therapy. It is therefore necessary to provide a treatment that will act quickly and ideally in a single dose. This points to a need to accurately and quantitatively determine the rate of drug action on malarial parasites with a priority to match or exceed the rate of action of artemisinin and its current combination therapies, the current gold standard in malaria treatment.

As discussed in Chapter 1, the rate of action of antimalarial drugs *in vitro* is usually determined by tracking the deterioration of parasite morphology during drug exposure in culture using Giemsa smears and light microscopy which is time-consuming, open to subjective interpretation and difficult to quantify confidently. The alternative PRR assay measures the ability of parasites to recover from exposure to drugs and is more definitive, but requires prolonged culturing and is limited in throughput. What is needed is a means to unambiguously quantify viable and non-viable/compromised parasites in cultures during drug exposure. To achieve this, this study explored the development of a novel bioassay using fluorescent vitality probes coupled with fluorescence microscopy which allowed the rate of drug action on malaria parasites to be assessed i) rapidly (in relation to current assay techniques) and ii) in a semi-quantitative manner.

The approach was to use fluorescent vitality probes in order to definitively distinguish between viable and compromised populations of drug treated parasites over specific time intervals. A dual method of staining was employed with the cytoplasmic staining of viable

cells with the readily fluorescent compound calcein, modified into a neutral molecule by the addition of an acetoxymethyl group (calcein-AM). The product can freely permeate cell membranes and subsequently be converted into its fluorescent counterpart via cytoplasmic esterases. These compounds are then retained by healthy cells due to their negative charge. The second stain is a nuclear stain with fluorescent probes with aromatic amino groups such as PI which interacts with nucleotides directly to produce red fluorescence. PI is negatively charged and cannot readily permeate cellular membranes. A loss of membrane integrity allows for the subsequent influx of fluorescent PI and it therefore acts as an indicator of cellular death and hence can be used to detect cellular degradation.

In addition to calcein-AM and PI, the ability of TMRE and hydroethidine to distinguish between viable and non-viable parasites was also determined. Since the probes are marketed for use in mammalian cells, fluorescence microscopy was used to first assess the behaviour of the probes in viable/compromised cultured mammalian (HeLa) cells. Subsequently, methods for staining cultured malaria parasites with the probes were optimised and used to assess their ability to detect viable/non-viable parasites by fluorescence microscopy. Based on the results, calcein-AM and PI were selected for further development. Parasites were exposed to a panel of standard antimalarial drugs and changes in the ratios of viable vs. non-viable parasites over time determined by fluorescence microscopy. The rates of these changes were consistent with the known properties of the individual drugs. This study developed a novel bioassay that utilizes these fluorescent probes in a manner that allows the rate of action of a panel of standard antimalarials to be ranked in relation to each other.

2.2 - Materials and Methods

2.2.1 - Thawing Cryopreserved Cultures

Cultures were thawed from frozen *P. falciparum* stocks previously prepared by Mr. J. Njunge. Cryotubes were thawed by hand until completion and parasite suspensions were then transferred to a 50 mL Falcon tube. Thawing Solution A (Appendix 1) was added dropwise, under constant agitation, to the thawed culture at 0.2x the volume of parasite suspension. Thawing Solution B (Appendix 2) was added at 2x the volume of the parasite suspension dropwise and under agitation. After being allowed to stand for 3 min the suspension was then centrifuged at 460 *g* for 3 min and the supernatant aspirated off. Fresh, uninfected RBC's (Appendix 3) were then added to the infected RBC's at a ratio of 1:1 and 10 mL of complete culture medium (CM - Appendix 4) was added to the suspension. The freshly prepared culture was then gassed using special gas mixture (5% CO_2 ; 5% O_2 ; 90 % N_2) and transferred to a T25, solid capped, culture flask. The flask was then placed in a 37^{o}C incubator and agitated at 45 rpm.

2.2.2 - Maintenance of *P. falciparum* 3D7 Parasite Cultures

Cultures were maintained according to an adaptation of the procedure by Jensen (1977) and scaled to 20 mL in a T75, solid capped, culture flask, and maintained at approx. 3 % parasitaemia and 3 % haematocrit. Cultures were removed daily from their respective non-filter cap culture flasks (Porvair) and placed in 50 mL Falcon tubes. After centrifugation at 460 *g* the spent media was aspirated off and a Giemsa (Appendix 5) stained smear was prepared from the pelleted parasitized RBC's. Smears were made to assess parasitaemia and quality of parasite morphology using a light microscope and 10 distinct fields of view. This was judged in accordance with requirements for current experimentation needs. As required, a portion of the cultured RBC pellet was discarded and replaced with fresh RBCs to maintain

the parasitaemia and haematocrit. Remaining parasitized RBC's were returned to the T75 and 20 mL of fresh CM was added. The flask was then gassed and placed in the incubator at 37^{o}C.

2.2.3 - Synchronization of Parasite Cultures

To ensure that all parasites in each respective culture are at the same morphological and metabolic life stage it was necessary to synchronize the culture every two weeks.

All experimentation was conducted using mid- to late-stage trophozoites. As malaria parasite culture provides a mixture of ring, trophozoite and schizont life stages it is necessary to treat the culture with sorbitol so as to induce synchronous development (Lambros and Vanderberg, 1979). Cultured RBC's containing predominantly ring stage parasites were centrifuged at 460 *g* for 3 min and the pellet was resuspended in 5% sorbitol (Appendix 6) for 5 min at ambient temperature. The cellswere then washed, resuspended in CM and transferred to a T75 close capped culture flask and gassed using the special gas mixture. The flask was replaced into a 37^{o}C incubator and agitated at 45 rpm.

2.2.4 - Saponin Lysis

A synchronous parasite culture of trophozoites was removed from culture and centrifuged at 460 *g* for 3 min after which the media was aspirated. The pellet was resuspended in 1 mL of 0.1 % saponin in PBS buffer for 5 min. The solution was centrifuged at 1050 *g* for 3 min and washed with CM.

2.2.5 - Parasite Enrichment

In order to obtain a culture devoid of all ring stage parasites and uninfected RBC's, an enrichment using Percoll was performed. A parasite culture of primarily trophozoite and schizont-stage parasites was centrifuged at 730 *g* for 3 min and the pellet transferred to the top of a 1 mL 60 % Percoll solution(Appendix 7) in an Eppendorf tube. The tube was then centrifuged at 8609 *g* for 20 min resulting in the formation of three distinct layers. The bottom layer containing uninfected and ring stage parasite infected RBC's, the centre layer containing the remaining Percoll solution and the upper most brown layer containing all trophozoite and shizont stage parasite-infected RBC's. The brown layer was removed and transferred to a sterile 15 mL falcon tube. CM was then added to the pellet slowly along with fresh RBC's in such a ratio as is required for routine culture or alternately experimentation. The culture was then returned to a T75 close capped culture flask, was gassed and placed in an incubator at 37^{o}C and agitated at 45 rpm.

2.2.6 - Fluorescent Vitality Probe Staining of Mammalian Cells

Stock solutions of each probe were obtained from Sigma-Aldrich (Steinheim, Germany) calcein-AM and PI were solubilised in double distilled water (ddH_2O) while TMRE and hydroethidine were solubilised in dimethyl sulfoxide (DMSO) after which they were stored in tubes in the dark. HeLa cells (Cellonex) were cultured under sterile conditions in Dulbecco's modified eagle medium (DMEM) with Ultralgutamine and 4.5 g/L glucose (Lonza, Belgium) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (penicillin/streptomycin/amphotericin B) in T75 flasks with vented caps in a 37^{o}C, 5 % CO_2 incubator. When cells were near confluence, they were detached from the flask in trypsin-EDTA (Lonza, Belgium), pelleted in a 15 mL tube and resuspended in culture medium. After counting the cells using a light microscope and haemocytometer, 10^5 cells per well were

transferred into the wells of a 24 well clear plastic culture plate containing 12 mm round glass coverslips and allowed to incubate in a 37^{o}C, 5 % CO_2 incubator overnight. The following day the culture medium was removed and the cells were washed twice in PBS buffer. To simulate compromised cellular vitality, respective wells were incubated in 100 µM of gramicidin S in PBS for 30 min. Wells containing healthy cells remained under standard culture conditions for this time period. The PBS (and drug if applicable) was then removed and the respective fluorescent probes (in PBS) were added to the cells according to Table 1 and allowed to incubate at 37^{o}C for the indicated times.

Table 1: Respective time and concentrations adopted for each fluorescent probe staining of HeLa cells.

Fluorescent probe	**Concentration (µg/mL)**	**Time (min)**	**Reference**
Propidium Iodide	20	30	Hui, Z. 2012
DAPI	2	15	Saha *et al.*, 2013
Hydroethidine	3	10	Forkink *et al.*, 2010
Tetramethylrhodamine	0.05	10	Dikov and Hahn, 2013
Calcein-AM	5	30	Schoonen *et al.*, 2005

Staining solutions were then removed and cells were washed twice with PBS buffer. In cases where more than one probe was used in the same well the incubation time was extended to accommodate both stains. After removal of the PBS the cover slip was transferred to a microscope slide and observed on a ZEISS Axiovert fluorescent microscope using a 40x objective and the UV (DAPI), blue (calcein-AM) and green (PI, TMRE, hydroethidine) excitation channels based on the excitation and emission spectra in Table 2.

Table 2: Excitation and Emission spectra of the fluorescent vitality probes used in the study.

Fluorescent probe	**Excitation/Emission maxima (nm)**
Propidium Iodide	535/617
DAPI	358/461
Hydroethidine	535/610
Tetramethylrhodamine	548/573
Calcein-AM	490/520

2.2.7 - Fluorescent Vitality Probe Staining of *P. falciparum* Malaria Parasites

Sufficiently synchronised parasite cultures were maintained between 2-5 % parasitaemia for staining and fluorescence microscopy visualization purposes. A T75 culture flask containing 20 mL of total culture was centrifuged at 460 *g* for 3 min and the supernatant removed. From the parasitzed RBC pellet, 20 uL was removed and placed into an Eppendorf tube containing 200 uL of CM. To simulate compromised cellular vitality, respective tubes were incubated in 200 pM of gramicidin D for 30 min. Eppendorf tubes containing healthy cells remained under standard culture conditions for this time period. Once the incubation time period had elapsed the cells were washed once in CM and 200 µL of staining solution was added in CM. The cells were gassed and allowed to incubate at 37^{o}C for the relevant time. Staining concentrations and incubation times were adapted and optimized according to patterns observed in the mammalian cell staining. Final optimized staining parameters are provided in Table 3.

Table 3: Respective time and concentrations adopted for each fluorescent probe staining of *P. falciparum* parasites.

Fluorescent probe	**Concentration (µg/mL)**	**Time (min)**
Propidium Iodide	3.3	15
DAPI	0.7	15
Hydroethidine	1	10
Tetramethylrhodamine	0.05	10
Calcein-AM	1	30

Staining solutions were then removed and cells were washed twice with CM. In cases where more than one probe was used in the same tube the incubation time was extended to accommodate both stains. After removal of the CM a small amount of the pellet was transferred to a microscope slide, covered with a coverslip and observed on a ZEISS Axiovert fluorescent microscope using a 100x objective.

2.2.8 - Drug IC_{50} values using the Plasmodial Lactate Dehydrogenase Assay

To obtain dose response curves for test compounds and obtain drug IC_{50} values pLDH assays were conducted with a method adapted from Makler *et al.,* (1993). Working solutions were made from drug stock solutions (Appendix 8) and a 2 % haematocrit, 2 % parasitaemia culture containing healthy, trophozoite stage, *P. falciparum* 3D7 parasites was used for the assay. Control solutions comprising a negative control of unparastized RBC's and a positive control of healthy, untreated, parasitzed RBC's were added to a 96 well plate at 100 µL per well in triplicate. Three-fold serial dilutions of the test compounds in CM were added to the same plate at twice the final test concentrations and an equal volume of parasite suspension (100 µL) was added to each well. The plate was transferred to an air tight container, gassed

using a special gas mixture (5% CO_2 ; 5% O_2 ; 90 % N_2) and allowed to incubate at 37^{o}C for 48 hours. Post incubation the cells were resuspended and 20 µL of each sample was transferred to a new clear bottom 96 well plate pre-loaded with 100 µL of Malstat solution (Appendix 9). In addition, 25 µL of NBT/PES solution (Appendix 10) was added to each well and any air bubbles were removed. The plate was left to develop in the absence of light before the absorbance was measured at 620 nm in a plate reader. Absorbance values were then used to calculate the percentage of parasite viability in each respective well according to the following equation:

$$\%\ Parasite\ viability = \left[\frac{(Absorbance\ of\ the\ sample - Mean\ Absorbance\ of\ background\ control)}{Mean\ absorbance\ of\ postivive\ control - Mean\ absorbance\ of\ background\ cotrol}\right] x\ 100$$

To obtain dose response curves for the test compounds, median inhibition concentrations (IC_{50} values) were calculated using GraphPad Prism (v5.02, san Diego California, USA).

2.2.9 – Test Compound Assessment Using the Fluorescent Staining *P. falipaum* Procedure.

As previously described synchronised parasite cultures were maintained between 2-5 % parasitaemia. A T75 culture flask containing 20 mL of total culture was centrifuged at 460 g for 3 min and the supernatant removed. From the parasitzed RBC pellet, 20 uL was removed and placed into an Eppendorf tube containing 200 µL of CM. Test compounds were added at 10x their IC_{50} concentrations as follows: artemisinin – 160 nM; doxycycline – 120 µM; chloroquine – 125 nM; mefloquine – 95 nM. After drug compound addition, the tubes were gassed and placed in a 37^{o}C heating block for 8 hours. Once the incubation time period had elapsed the cells were washed once in CM and 200 µL of staining solution was added after which they were gassed and allowed to incubate at 37^{o}C for the relevant staining time.

Staining concentrations were as per the indications in Table 3. Staining solutions were then removed and cells were washed twice with CM. In cases where more than one probe was used in the same tube the incubation time was extended to accommodate both stains. After removal of the CM a small amount of the pellet was transferred to a microscope slide and observed on a ZEISS Axiovert fluorescence microscope.

2.3 - Results

2.3.1 - Assessment of Fluorescent Vitality Probe Staining in Mammalian Cells

The fluorescent vitality probes selected for this study are predominantly marketed for mammalian cell use and have been used extensively with fluorescence microscopy, flow cytometry and fluorimeter spectroscopy. Although shown to be able to stain their respective cellular elements in other organisms including bacteria (Vesey *et al.,* 1994), and protozoa (Mossallam 2010) these probes are more widely utilized in mammalian cell studies.Although the probes selected have been used in *Plasmodium* parasites, this has been as a diagnostic tool and these studies often make use of flow cytometry instead of fluorescence microscopy.It was therefore necessary to assess their staining patterns in mammalian cells prior to their use in *P. falciparum* malaria parasites in order to understand their innate staining patterns.

Briefly, HeLa cells were removed from culture and transferred to a 24-well clear plastic culture plate containing a cover slip in each well and allowed to incubate at 37^{o}C and 5 % $CO_2$for 24-hours. This would allow the cells to adhere and grow on the coverslip for subsequent staining and microscopy. After two washes in PBS 200 µL of the respective staining solution was added to a well containing a cover slip and incubated at 37^{o}C as follows – calcein-AM (5 µg/mL) for 30 min; hydroethidine (3 µg/mL) for 10 min; TMRE (0.05 µg/mL) for 10 min. Staining solutions were then removed and the cells were washed twice

with PBS after which the cover slip was placed onto a microscope slide and observed on a ZEISS Axiovert fluorescent microscope.

In the instance that it was necessary to simulate compromised cellular vitality (to control for a drug treated culture), respective wells were incubated in 100 µM of Gramicidin-S for 30 min prior to staining for 30 min. Gramcidin S was selected due its significant toxicity to human cancer cells in culture for concentrations exceeding 70 µM as well as its rapid activity (Nir-Paz *et al*., 2002).

2.3.1.1 - Calcein-AM

The acetomethoxy ester of calcein, calcein-AM, was seen to freely permeate cellular membranes and was cleaved to green fluorescent calcein. The probe was then retained well by healthy cells and a marked decrease was observed in gramicidin S treated cells (Figure 8). The extent of calceinfluorescence intensity was observed to disappear as cellular vitality was compromised by drug action. DAPI positive cells were observed in greater quantity after drug treatment which coincided with a decrease in calcein staining. There also appeared to be no dual staining of any cells as each stained cell contained either DAPI or calcein.

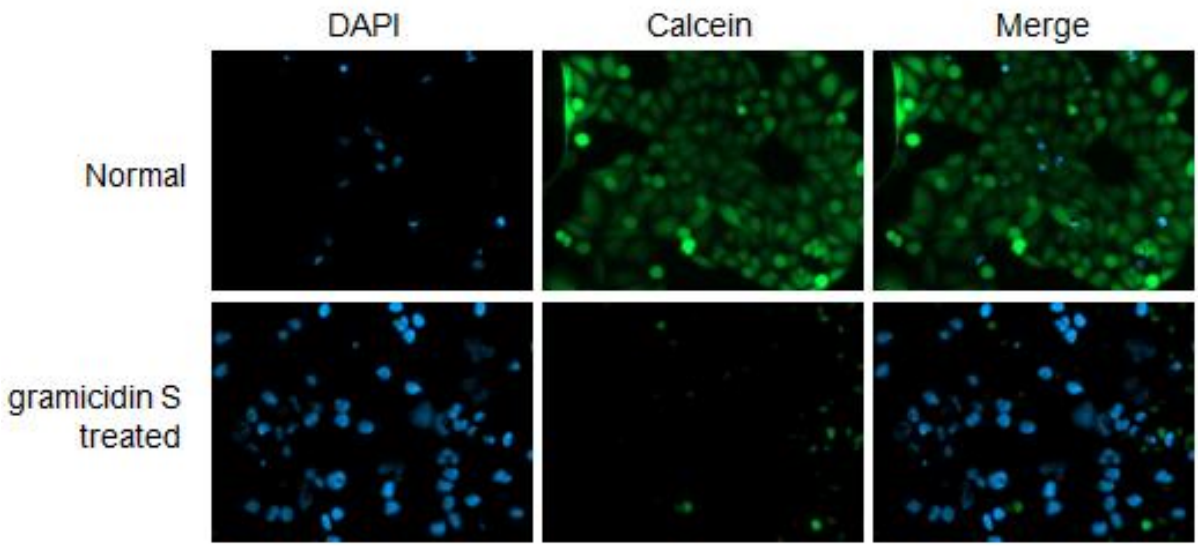

Figure 8: Calcein (5 µg/mL) and DAPI (2 µg/mL) staining of Hela cells treated with 100 µM gramicidin S for 30 min alongside untreated cells. To simulate healthy cell conditions the 'Normal' cells were incubated in media at 37°C and 5 % CO_2 during the period of drug incubation for the 'gramicidin S treated' cells. Total probe incubation time was 30 min to allow for both optimal DAPI and calcein-AM staining.

Drug treated cells showed a significant increase in DAPI staining and a distinct decrease in fluorescent calcein.The staining pattern of the cleaved calcein was observed to have no particular subcellular localization and instead was spread evenly through the cytoplasm and nuclei of the HeLa cells.

2.3.1.2 - Propidium Iodide

Although requiring minor optimization which included adjustments to staining concentration and time, PI proved useful in acting as a vitality probe and showed good fluorescent intensity in the presence of drug treated, dead cells (Figure 9). It was seen to have minor retention in a healthy cell culture although the signal was weak and could have been an indication of recently membrane comprised cells that were still capable of minor cellular function.

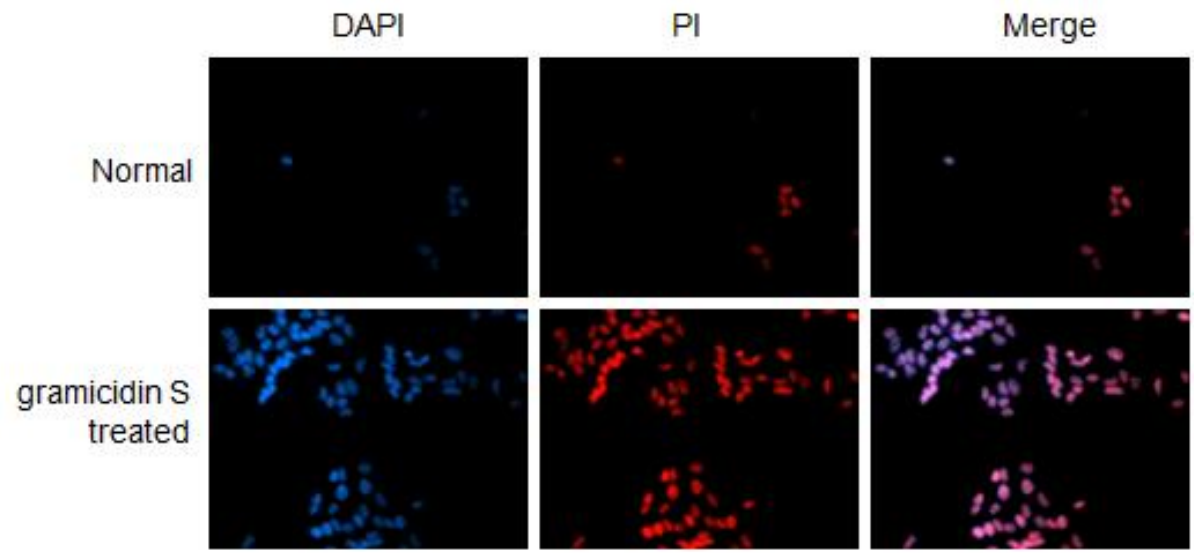

Figure 9: PI (20 µg/mL) and DAPI (2 µg/mL) staining of Hela cells treated with 100 µM gramicidin S for 30 min alongside untreated cells. To simulate healthy cell conditions the 'Normal' cells were incubated in media at 37°C and 5 % CO_2 during the period of drug incubation for the 'gramicidin S treated' cells. Total probe incubation time was 30 min to allow for both optimal DAPI and PI staining.

It was observed that only cells that could be classified as 'dead' (but not entirely lysed) were capable of retaining the probe and as such drug treated cells showed an increase in DAPI staining and a localized increase in PI staining. The fact that the DAPI and PI staining coincided exactly could be expected as they both stain the DNA in cell nuclei. It also suggests that DAPI acts as a vitality probe for HeLa cells, only accumulating in the nuclei of compromised cells.

2.3.1.3 - TMRE

Probing HeLa cells with TMRE provided many challenges. In relation to the PI and calcein-AM studies there appeared to be significantly fewer 'dead' cells after Gramicidin-S treatment which would suggest the drug was not affective in the TMRE sample. The drug treated sample showed an increase in DAPI 'ghost-like' staining of a large proportion of the HeLa cells and a minor increase in dead cells with the same fluorescence intensity as observed in

the PI and calcein-AM drug treated cultures (Figure 10). Subsequent TMRE samples yielded similar results however.

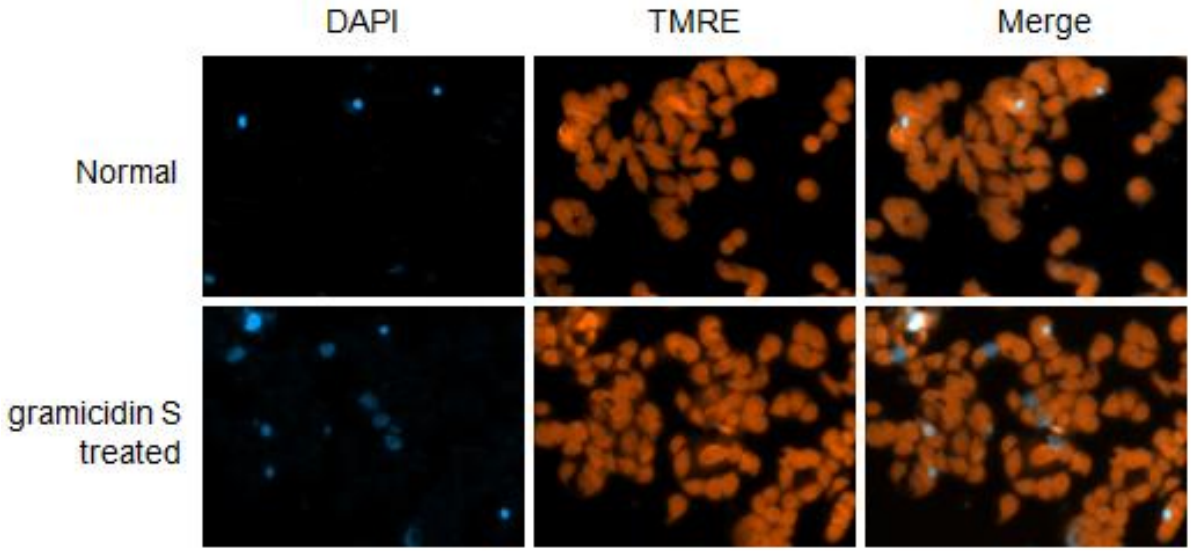

Figure 10: TMRE (0.05 µg/mL) and DAPI (2 µg/mL) staining of Hela cells treated with 100 µM gramicidin S for 30 min alongside untreated cells. To simulate healthy cell conditions the 'Normal' cells were incubated in media at 37^{o}C and 5 % CO_2 during the period of drug incubation for the 'gramicidin S treated' cells. Total probe incubation time was 15 min to allow for both optimal DAPI and TMRE staining.

TMRE staining is observed in both the drug treated and normal culture samples with localization with the DAPI stained 'ghost-like' cells indicating these cells may be slightly affected by drug treatment but still maintained sufficient cellular function and structure to sustain the probe. The more intensely fluorescent DAPI-positive cells showed no localization with TMRE indicating that TMRE was incapable of being retained in these dead cells. Furthermore there was no indication of any localization to the mitochondria suggesting TMRE only stains viable cells albeit the cytoplasm and mitochondria included.

2.3.1.4 - Hydroethidine

The uptake of hydroethidine within the Hela cells was similar to that found with TMRE. In contrast to TMRE staining however there was a significant increase in DAPI-positive cells post drug treatment. DAPI staining appeared minimal in healthy HeLa cells and instead large scale hydroethidine fluorescence was observed for these cells. A significant increase in DAPI staining occurred in drug treated cells while hydroethidine staining appeared unchanged from the untreated cell sample (Figure 11). There appeared to be significant co-localization of the DAPI and hydroethidine probes in both the untreated and gramicidin S treated samples albeit in greater quantity in the drug treated sample.

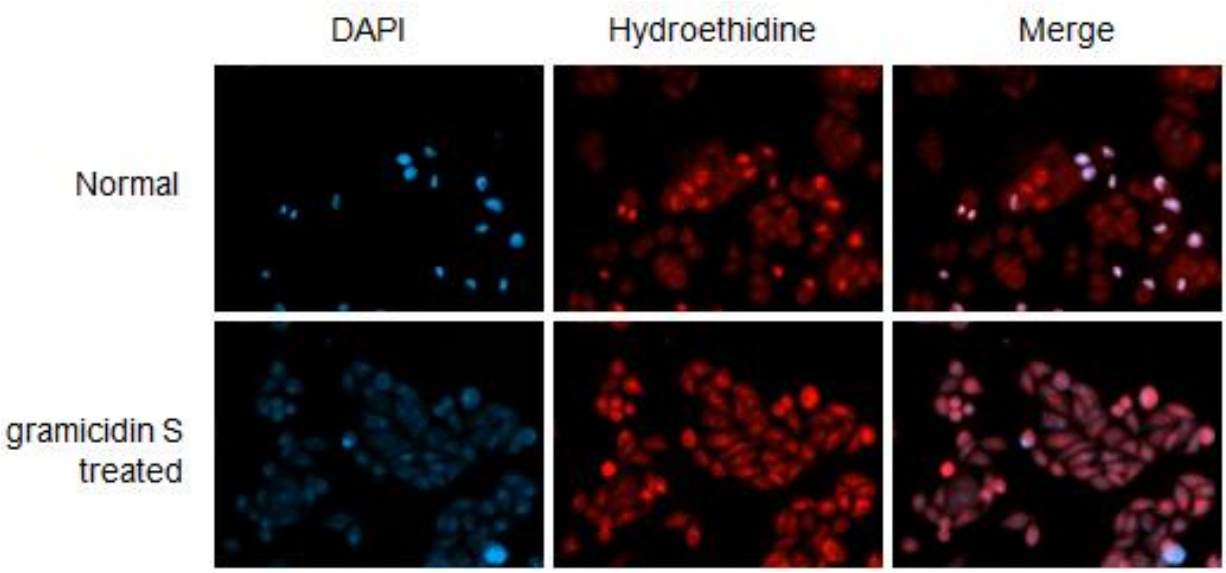

Figure 11: Hydroethidine (3 µg/mL) and DAPI (2 µg/mL) staining of Hela cells treated with 100 µM gramicidin S for 30 min alongside untreated cells.To simulate healthy cell conditions the 'Normal' cells were incubated in media at 37^{o}C and 5 % CO_2 during the period of drug incubation for the 'gramicidin S treated' cells. Total probe incubation time was 15 min to allow for both optimal DAPI and Hydroethidine staining.

Drug treated cells showed large scale DAPI fluorescence as well as co-localization with hydroethidine. Untreated cells showed minor DAPI fluorescence coupled with high fluorescence intensity in hydroethidine positive cells.

2.3.2 - Fluorescent Vitality Probe Staining and Optimization in *P. falciparum.*

Although the probes selected for use in the development of the vitality assay are predominantly marketed for mammalian cell use, they have all been used to some extent in parasites, although often as a means to quantify parasite numbers after drug treatment in end-point drug efficacy assays, as opposed to the rate of action assays as proposed in this study. Having optimized staining in mammalian cells and assessed parasite staining procedures in literature, draft staining protocols for *P. falciparum* were prepared and tested in pilot assays. This was required to assess the extent of fluorescence intensity in viable and non-viable parasite cells and evaluate if these staining patterns were comparable to those obtained from the mammalian cell study. It was observed that based on the morphological and chemical differences in *P. falciparum* cells, optimization was required for reliable staining methods could be reached, again focusing on concentration and duration of probe staining as well as concentration and duration of drug treatment where applicable.

Optimized staining protocols were as follows: a parasite culture of between 2-5 % parasitaemia was pelleted and 20 uL removed and placed into an Eppendorf tube along with 200 uL of complete media. For untreated cell samples, 200 µL of the respective staining solution was added and incubated at 37^{o}C as follows – calcein-AM (1 µg/mL) for 30 min; Hydroethidine (1 µg/mL) for 10 min; TMRE (0.05 µg/mL) for 10 min. Staining solutions were removed and the cells were washed twice with complete media after which a small volume of the pellet was transferred to a microscope slide under a cover slip, and observed on a ZEISS Axiovert fluorescence microscope.

In the instance that it was necessary to simulate compromised cellular vitality (to control for a drug treated culture), respective tubes were incubated in 200 pM of gramicidin D for 45 min prior to staining.

2.3.2.1 - Calcein-AM

Calcein-AM was observed to readily enter and be retained by healthy parasites. In contrast, membrane compromised parasites post gramicidin D treatment were unable to retain the probe and hence exhibited no fluorescence. Staining patterns covered the entirety of the parasite matrix excluding only the food vacuole which showed no staining (Figure 12). The fluorescence intensity was reduced in schizonts when compared with trophozoites while DAPI fluorescence intensity in parasite nuclei remained equal in both untreated and treated test samples.

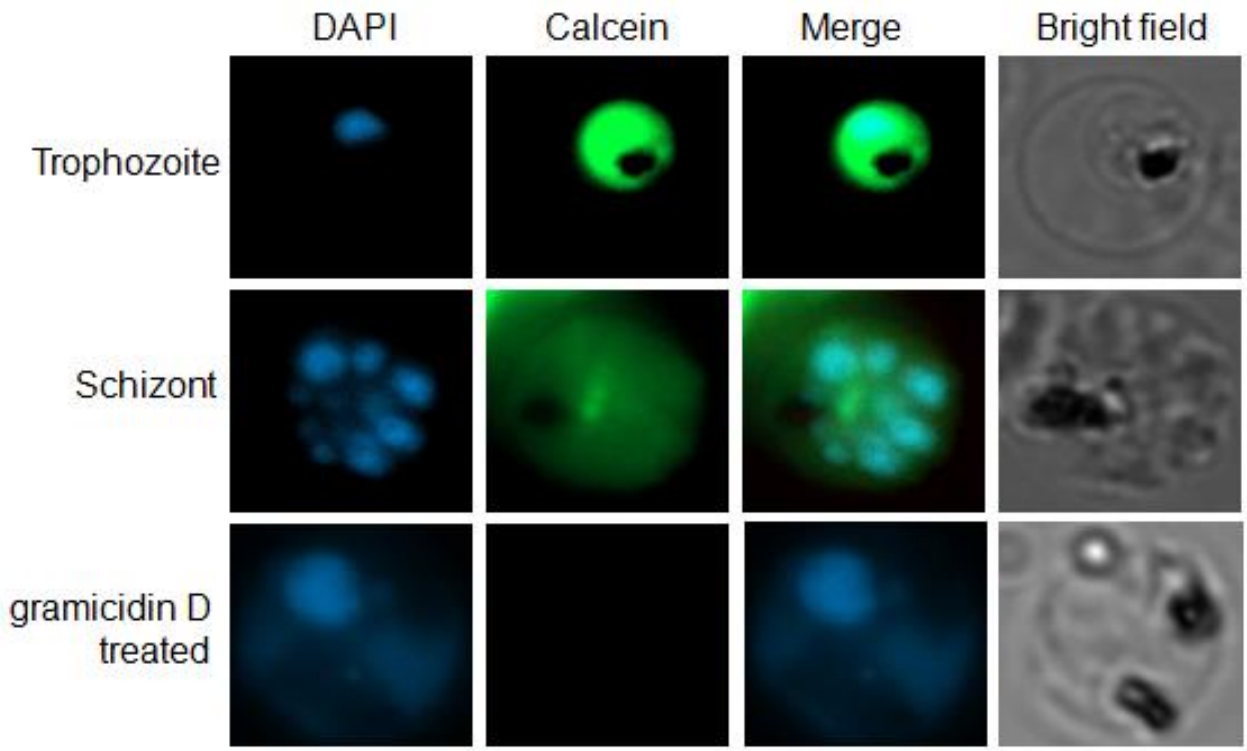

Figure 12: Drug treated parasite are indicative of staining patterns observed after gramicidin D (100 pM) treatment for 45 min. The dark, unstained region in the trophozoite and schizont stage parasites represents the food vacuole, as denoted by the prominent haemozoin crystal in the bright field image.

Calcein-AM staining in *P. falciparum* parasites showed wide fluorescent coverage of the parasite matrix and absence from the parasite food vacuole. Fluorescent signals observed in healthy trophozoites were found to be of sufficient quality at 1 µg/mL, a five times decrease from that required for mammalian HeLa cells in this study. Schizont staining followed similar patterns to trophozoite although staining intensity was decrerased in comparison. It was further observed that the calcein staining was not limited to the parasite, but instead stained the cytoplasm of infected and uninfected RBC's (Figure 14). This would naturally have a subjective impact on users who may mistakenly classify a RBC as a healthy parasite. In addition, any fluorescent measurements using a flow cytometer or plate reader would deduce a signal from a RBC as a parasite fluorescent signal. An additional saponin lysis step (a detergent capable of selectively lysing RBC membranes, leaving parasites intact) was

inclucded into the calcein procedure to reduce the variation contributed by RBC staining (Figure 13).

The addition of saponin was initiated using a synchronized parasite culture containing 2 % trophozoite parasitaemia. The culture was pelleted and resuspended in 1 mL of 0.1 % saponin in PBS for 5 min. The trophozoite culture absent of RBC's was subsequently pelleted and washed in complete media before staining with calcein-AM (1 µg/mL).

Calcein-AM staining of a sample of uninfected RBC's yielded RBC's with the acetoxymethyl ester being cleaved in the cytoplasm and resultant large scale fluorescent calcein in the RBC matrix (Figure 13A). This staining pattern was not dissimilar in infected RBC's. Panel B is a magnified image of a trophozoite-infected RBC with calcein fluorescence across the entirety of the RBC cytoplasm and a slightly more intensely stained trophozoite parasite. With the inclusion of saponin treatment prior to visualization parasites are observed to retain the calcein probe while the RBC's are disrupted to a significant enough extent that their fluorescence is all but eliminated (Figure 13C).

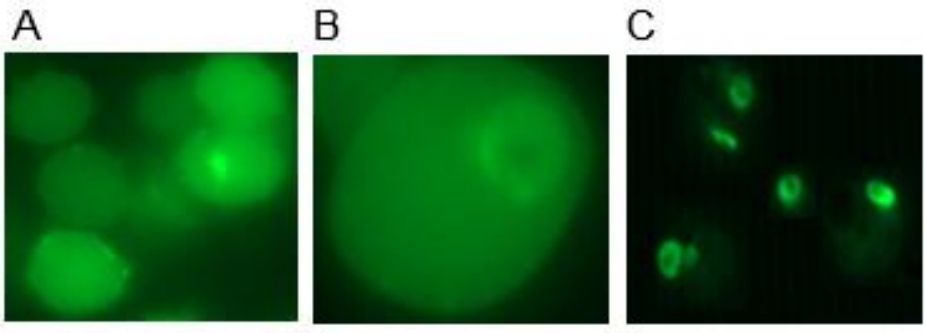

Figure 13: Calcein-AM (1 µg/mL) and fluorescent patterns in A – unparasitized RBCs, B – parasitized RBCs and C – saponin (0.1 % saponin) lysed parasitized RBC's. Note that panels A and C are at 1000x magnification, while panel B is enlarged to show a single parasitized RBC.

In parasitized RBCs the extent of the signal encompassed the entirety of the RBC in such a manner that it was difficult to distinguish the parasite from the RBC matrix. Post saponin

treatment the parasites are less open to subjective misinterpretation as their cellular morphology and size in general is easily distinguishable from RBCs as shown in panel C where brightly stained trophozoites are present and only negligible fluorescence remains in the surrounding lysed RBCs.

2.3.2.2 - Propidium Iodide

In contrast to calcein-AM staining, PI fluorescence was only observed once parasite membrane viability was sufficiently compromised. This required substantial optimization in order to obtain a gramicidin D concentration that provided enough of an impact on the cellular membrane to observe a PI signal while still maintaining enough structural integrity such that the signal was retained long enough to provide respectable fluorescent images and parasites were still morphologically intact and distinguishable under bright field.

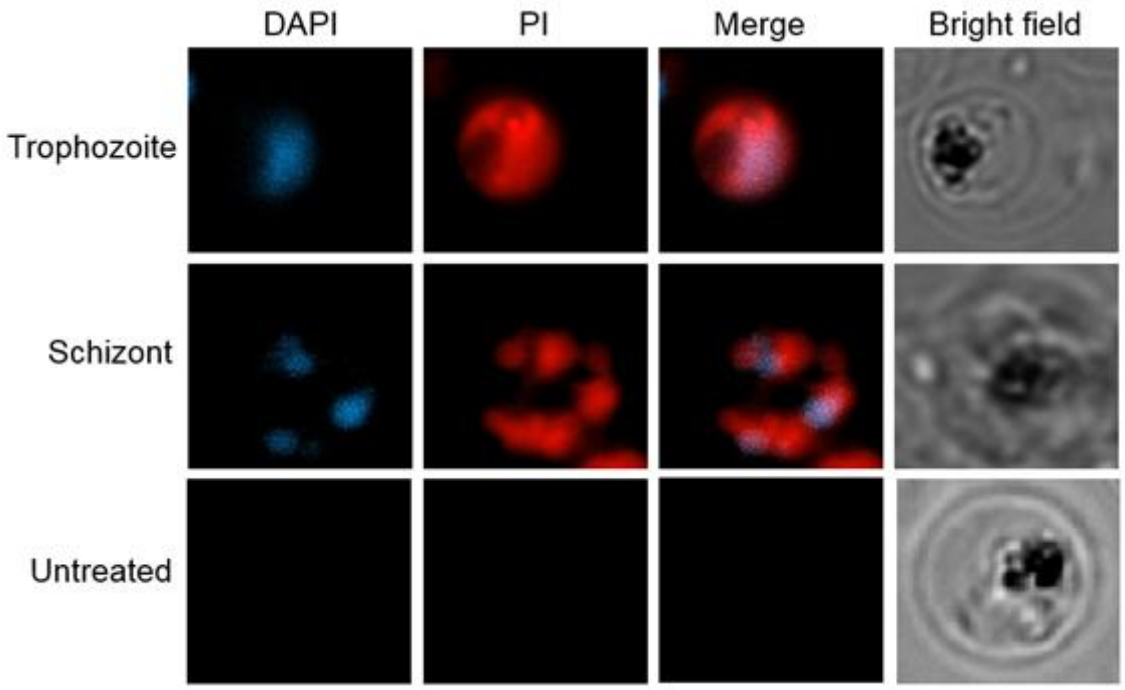

Figure 14: PI (3.3 µg/mL) and DAPI (0.7 µg/mL) staining of *P. falciparum* malaria parasites after a 45 min incubation in 100 pM gramicidin D prior to a 15 min stain.

A significantly lower concentration of PI was required for adequate parasite staining (3.3 µg/mL) as opposed to mammalian cell staining (20 µg/mL) with just over a six times lower concentration required. This was similar to what was observed when moving from mammalian staining to parasites using calcein-AM. Consistent with what was found with mammalian cells, no PI or DAPI staining was observed in healthy, untreated parasites (or RBCs), while significant PI accumulation was seen in gramicidin D treated trophozoites and schizonts (Figure 14). Peculiarly, unlike mammalian cells, PI staining was not only limited to the parasite nucleus (denoted by DAPI staining), but found throughout the parasite cytoplasm, particularly in the trophozoite stage (Figure 14, top row).

An interesting discovery was that PI appeared to stain the matrix of healthy merozoites,the small invasive stages of the parasite that rupture from infected RBCs following completion of the parasite life cycle (Figure 15) whereas calcein was not retained in these cells. Note that the cells were untreated. The observed strong PI staining and lack of calcein could provide false positives for nonviable parasites. In probing viable vs. non-viable parasites using PI and/or calcein staining, care should be taken not to include merozoites in the analysis.

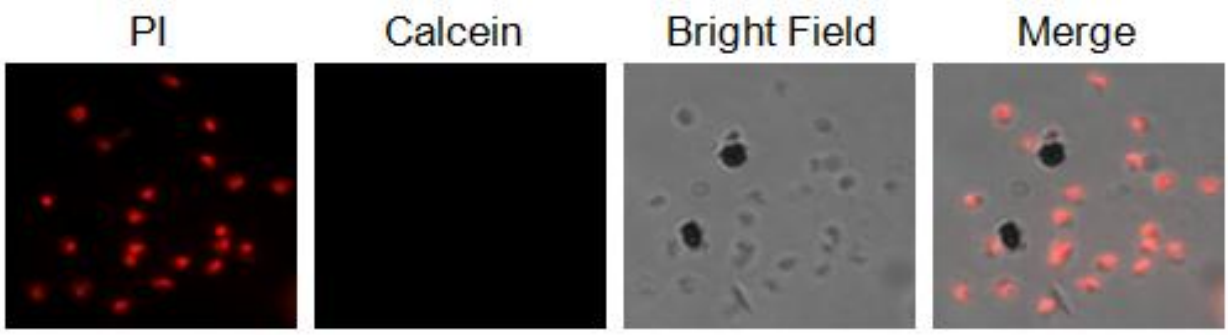

Figure 15: PI (3.3 µg/mL) and calcein-AM (5 µg/mL) staining of merozoite-stage *P. falciparum* 3D7 parasites. Parasites were allowed to grow past the trophozoite and schizont life stage in normal culture conditions and then pelleted, stained and observed under an Axiovert fluorescence microscope at 1000x magnification to observe staining patterns in merozoite stage cells. The image shows a large number of free-floating merozoites and two dark hemozoin crystal remnants of schizonts that had completed merozoite formation and release.

2.3.2.3 - TMRE

In this study it was observed that TMRE was able to cross the membranes of healthy malaria parasites, however the stain was not definitively seen to localize to the parasite mitochondria as was anticipated. Similarly to mammalian cells the probe sequestered to the cellular matrix and showed consistent fluorescence intensity across the parasite (with the exception of the dark region occupied by the haemozoin crystal in the food vacuole). Drug treatment with gramicidin D did not provide any change in staining patterns compared to healthy parasites (Figure 16).

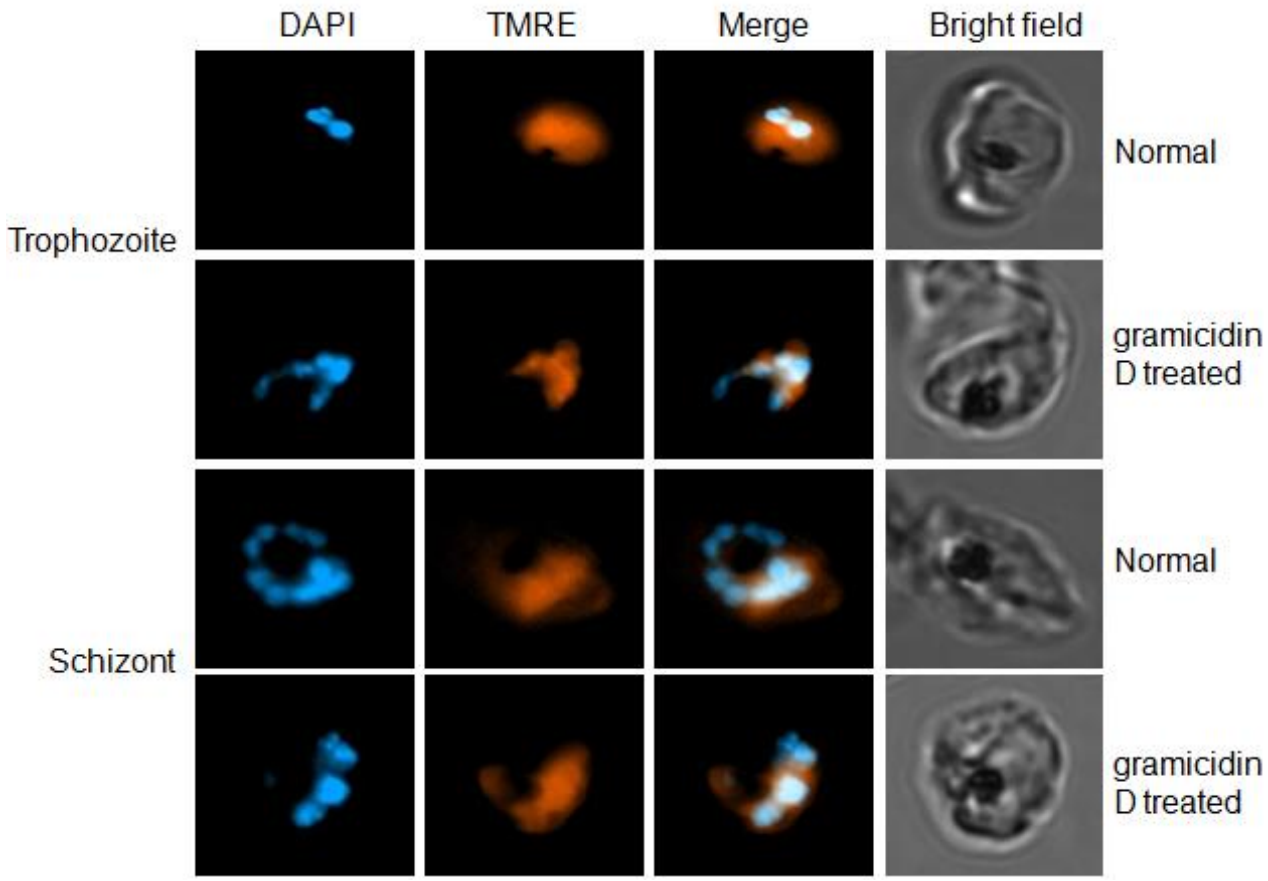

Figure 16: TMRE (0.05 µg/mL) and DAPI (0.7 µg/mL) staining of *P. falciparum* malaria parasites after a 10 min staining period. Drug treated parasites were subjected to a 45 min incubation in 100 pM gramicidin D prior to staining.

HeLa cell staining suggested no specific localization of TMRE and instead even staining throughout the cell was observed. TMRE did show some preferential staining of certain regions of malaria parasites (Figure 16) although it is unclear if this localization is mitochondria specific or what these regions represent. Regardless, unlike PI and calcein-AM, TMRE did not distinguish between treated and untreated gramicidin D parasites, both had similar staining patterns. Due to this consideration, it was decided to no longer continue pursuing TMRE as a suitable fluorescent vitality probe.

2.3.2.4 - Hydroethidine

Hydroethidine provided results similar to those seen in HeLa cells (with the exception that DAPI appeared unable to stain the nuclei of viable HeLa cells – in parasites, DAPI-staining is found in the nuclei of untreated and treated parasites in equal proportions). Drug treatment did not affect the hydroethidine fluorescence intensity in either drug treated or healthy HeLa cells and there was significant co-localization between DAPI and hydroethidine in the drug treated HeLa cells - a feature that was absent in healthy cells. Due to the ability of DAPI to also stain the nuclei of healthy parasites, extending the staining to parasites did not provide the same patterns and instead co-localization between DAPI and hydroethidine was seen irrespective of drug treatment (Figure 17).

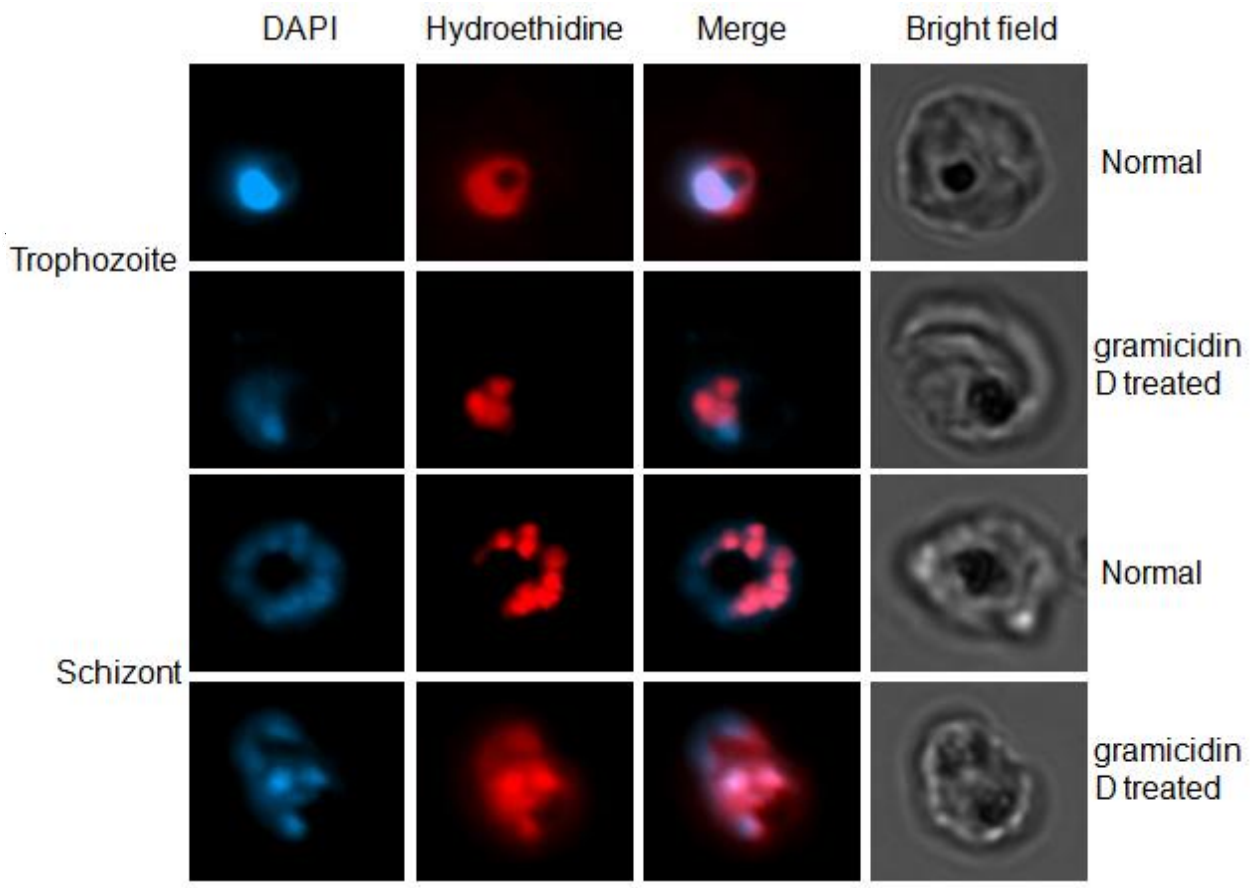

Figure 17: Hydroethidine (1 µg/mL) and DAPI (0.7 µg/mL) staining of *P. falciparum* malaria parasites after a 10 min staining period. Drug treated parasites were subjected to a 45 min incubation in 100 pM gramicidin D prior to staining.

Although used in various studies as a distinct indicator of viable cells it was observed that, similarly to HeLa cells, there was no decrease in fluorescence after drug treatment (Figure 17). The concentration of the probe was altered systematically, as well as the incubation temperature however no change in staining patterns was noted. Further assays were conducted with hydroethidine concentrationsranging from 3 to 6 µg/mL (which are more similar to concentrations used in published studies), but failed to yield differential staining of untreated and treated parasites. Hence hydroethidine was discarded as a suitable vitality probe for use in this study.

2.3.3 - pLDH assay to determine drug dose response curves and IC_{50} values

Having established that calcein-AM and PI were promising probes for distinguishing viable/non-viable parasites (while TMRE and hydroethidine were not) using gramicidin D as a model parasite compromising agent, the next step was to determine whether use of the probes could be extended to other conventional antimalarial drugs. Before the fluorescent vitality assay could be conducted on currently used antimalarials, it was necessary to confirm their efficacy against the lab strain of *P. falciparum* 3D7 parasites and to obtain a guideline of the concentrations to be used in subsequent experiments. Drug concentrations resulting in 50 % parasite cytotoxicity (IC_{50}) were determined using the lactate dehydrogenase (pLDH) cytotoxictiy assay. This involves the pLDH-mediated conversion of APAD to APADH which subsequently reduces nitro blue tetrazolium, the product of which can be measured spectrophotometrically.

A portion of a 2 % haematocrit, 2 % parasitaemiaculture was removed from the medium via centrifugation and incubated individually with varying serial dilutions of drug compounds in a 96-well plate i.e. chloroquine, mefloquine, artemisinin, doxycycline and methylene blue. Control solutions encompassed a negative control of unparastized RBC's and a positive control of untreated, parasitzed RBC's. After a 48 hour incubation, pLDH activity in individual wells was measured to assess surviving parasites, as detailed in section 2.2.8. The IC_{50} values for the individualdrugs were then calculated from dose-response curves obtained from three separate experiments (Figure 19) nd tabulated (Table 4).

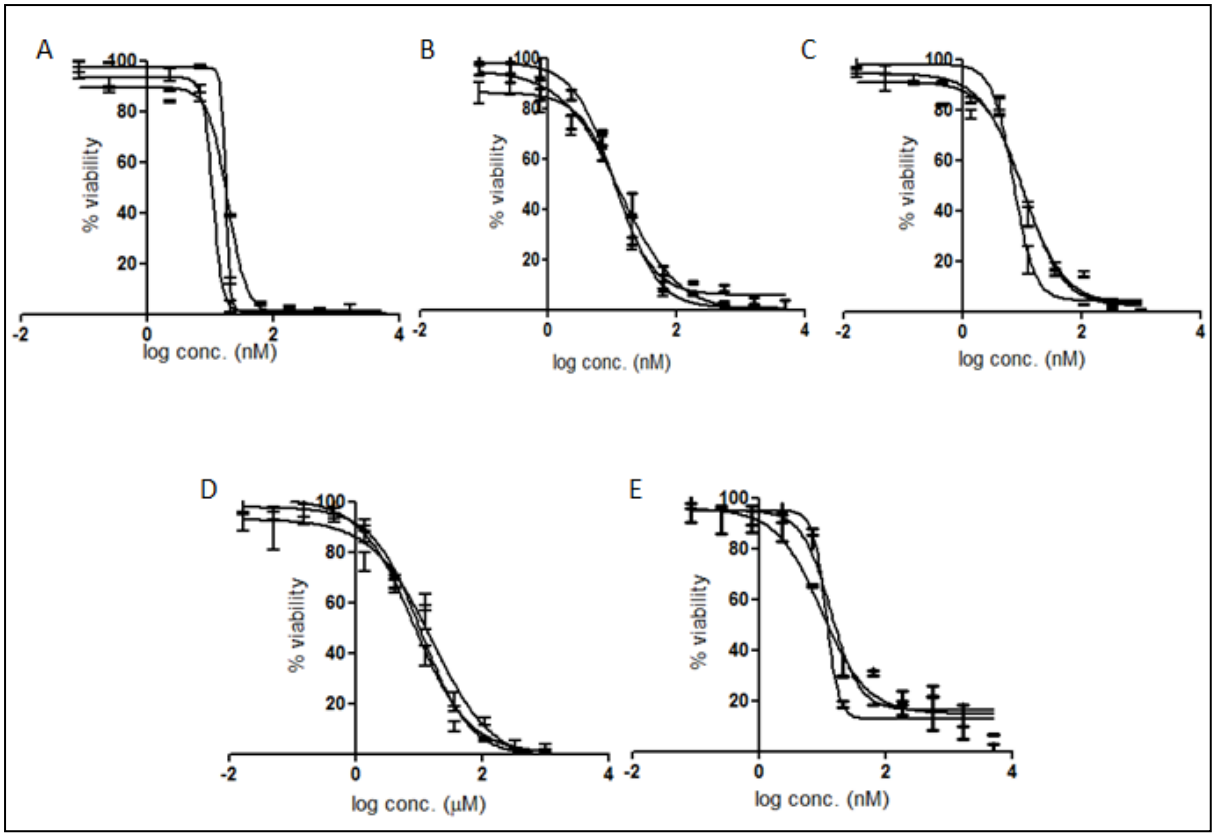

Figure 18: Dose response curves of the five *P. falciparum* 3D7 antimalarial drug compounds used in the study. pLDH assays were performed on three separate cultures and dose-response curvesplotted using GraphPad Prism v5.02. A: Artemisinin; B: Chloroquine; C: Mefloquine; D: Doxycycline; E: Methylene blue.

Table 4: Experimental IC_{50} values acquired from dose response curves of the five test compounds use in the pLDH assay in relation to cited literature values.

Drug	**Average Sample IC_{50}**	**Literature IC_{50}**	**Reference**
Artemisinin	16 nM	10 - 35 nM	Walcourt *et al.,* 2008; Amewu *et al.,* 2006; Bero *et al.,* 2009
Chloroquine	12 nM	8.5 - 14 nM	Moneriz *et al.,* 2011; Amewu *et al.,* 2006; Vivas *et al.,* 2008
Mefloquine	9 nM	6 - 19 nM	Vivas *et al.,* 2008; Aunpad *et al.,* 2009; Wong *et al.,* 2011
Doxycycline	12 µM	500 nM - 11 µM	Fall *et al.,* 2011; Dahl and Rosenthal, 2007; Achieng *et al.,* 2014
Methylene Blue	12 nM	2 - 21 nM	Okombo *et al.,* 2012; Akoachere *et al.,* (2005); Motau, 2015

All IC_{50} values in the panel were calculated to be in the same range with the lowest being mefloquine at 9 nM and highest being artemisinin at 16 nM. Importantly all IC_{50} values fell in the range of cited literature values.

2.3.4 - Optimized calcein-AM and PI Fluorescent Probe staining procedures with standard antimalarial compounds.

Due to the favourable vitality staining patterns observed with the calcein-AM and PI probes, further development of the bioassay involved combining probes with the drugs selected as test compounds in an effort to observe any change in staining patterns over an eight hour time period. These changes could then be qualitatively analysed using a primitive ‘cell count’ approach coupled with fluorescence microscopy such that the staining patterns could provide an indication of the rate-of-action of each drug used in the study.

Briefly, a synchronous parasite culture containing early stage trophozoites was pelleted and distributed into microfuge tubes containing complete media. Gramicidin D treatment was completed in parallel with each of the test compoundsat 10 fold IC_{50} concentration. After adding the respective compounds, the tube was gassed with 5% CO_2, 5% O_2, 90% N_2 and placed in a 37^{o}C heating block for 8 hours. The cells were then washed once in complete media and incubated in both calcein-AM and PI staining solutions at 37^{o}C after gassing. Excess probe was then washed off with complete media and a sample of the stained pellet was transferred to a microscope slide and observed on a ZEISS Axiovert fluorescence microscope.

It was observed that there was a clear decrease in the proportion of parasites stained with calcein and a corresponding increase in PI fluorescence moving down the respective panels representing parasites treated with the respective drugs (Figure 19). The most calcein fluorescence (viable parasites) was observed in the doxycycline treated sample through to artemisinin which had the largest observable PI fluorescence (non-viable parasites). Strikingly, PI and calcein fluorescence showed no co-localization and instead probed only dead and live cells respectively. These patterns were compared to untreated, healthy parasites and gramicidin D treated parasites which acted as positive (viable) and negative (non-viable) controls respectively. The positive control simulated optimal growth conditions over the eight hour period and this was evident in the staining pattern. Large scale calcein staining was observed with minimal PI fluorescence indicating an overall healthy culture (Figure 19, untreated). The minor PI staining observed comprised only 2 % of the total parasite population and can be attributed to general turnover of the parasite population. The untreated control appeared similar to the staining patterns observed in the doxycycline treated sample.

In contrast the negative control was used to provide an indication of fluorescent patterns in a parasite culture on the edge of collapse. The representative image shown in Figure 19 (gramicidin D) is figurative of what was observed in all fields of the treated sample - significant PI staining of all discernible parasites and minor calcein staining fragments or RBC artefacts. The percent of viable parasites reached a maximum of 2 % across all fields and hence provided insight into what to expect from a drug with rapid, potent efficiency. The negative control staining pattern most closely resembled that obtained from the artemisinin sample.

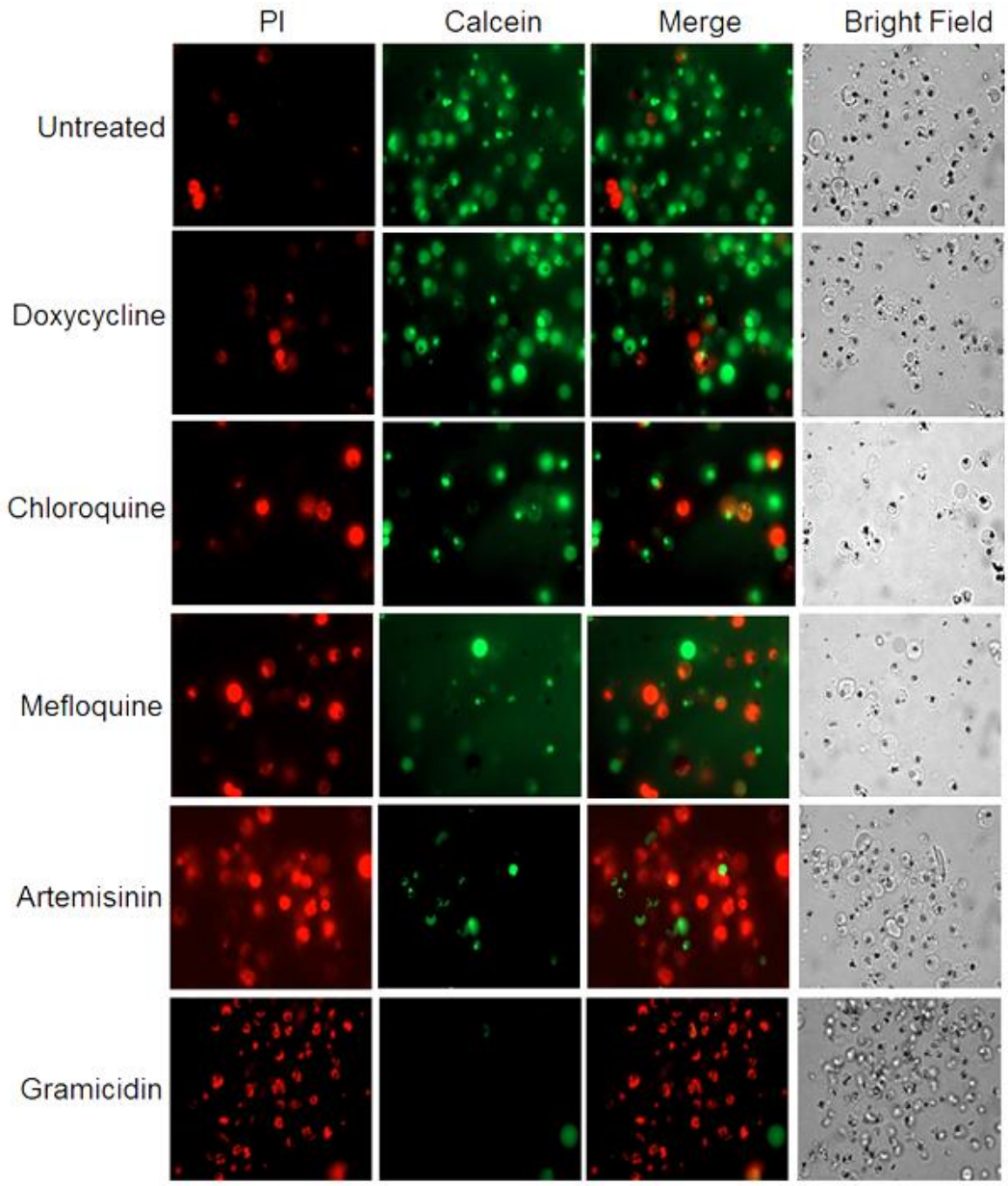

Figure 19: Dual probe staining of *P. falciparum* 3D7 parasites using PI (3.3 µg/mL) and calcein-AM (5 µg/mL). Untreated parasites were removed directly from normal culture conditions. Parasites were treated for 8 hours with doxycycline (100 µM), chloroquine (120 nM), mefloquine (90 nM) artemisinin (150 nM) and gramicidin D (100 pM) individually after which they were stained with PI and calcein-AM, treated with saponin to lyse RBCs, washed and viewed by fluorescence microscopy at 1000x magnification.

The total number of parasites that were counted in a field was completed using ImageJ v1.49 and the Cell Counter plugin. Total parasites were defined as both green fluorescent (calcein positive, viable) and red fluorescent (PI postitive, nonviable) cells using the bright field image as a reference. The ratio of green fluorescent cells to total parasite cell count was then calculated and final parasite viability was confirmed for each field of view across five distinct fields. The average parasite viability across the five fields for each drug treatment was used to generate Figure 20.

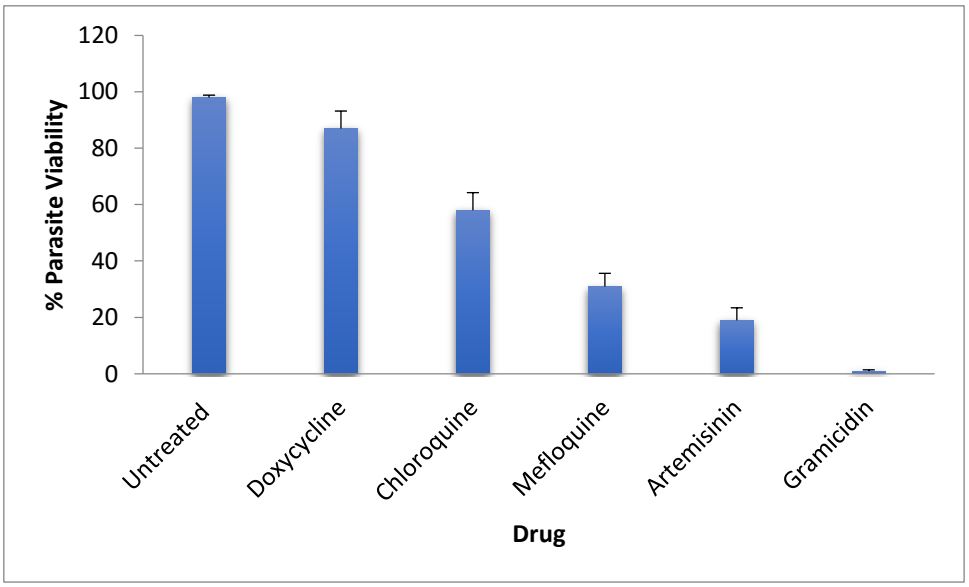

Figure 20: Rate of drug action for each test compound represented as percentage parasite viability visualized via fluorescence microscopy after an 8 hour drug treatment. Untreated parasites represent a healthy population at 98 % mean viability. Doxycycline (100 µM), chloroquine (120 nM), mefloquine (90 nM) and artemisinin (150 nM) represent 86 %, 60 %, 33 % and 13 % of parasite viability respectively. Gramicidin D (100 pM) treated parasites represent a decimated population at 2 % mean viability. Percentage parasite viability was calculated using ImageJ v1.49 and the Cell Counter plugin (De Vos, Academic Neurology, University of Sheffield). P-values were calculated for each remaining drug-treated viability in relation to the untreated control and in all instances $p < 0.01$ ($N = 5$).

Doxycycline, a member of the tetracycline antibiotic group, was observed to have the slowest rate of action by a considerable margin in relation to the other test compounds, yielding only a 12 % decrease in parasite viability in relation to the untreated control sample. It is evident

that doxycycline has a decidedly low impact on the parasite population over the 8-hour time period.

With a mean parasite viability of 60 %, chloroquine treatment resulted in morethan half of the parasite population still being metabolically active after the 8 hour time period. Removal of the drug at this stage would suggest that some of these parasites would lose viability post drug treatment while enough would survive to replicate and maintain the infection.

As a member of the quinoline family it would perhaps be expected that mefloquine would provide a similar mechanism and similar rate of drug action to chloroquine in malaria parasites. It was found that in this study that mefloquine was more rapidly acting in comparison to chloroquine with only 33 % of parasites remaining viable at the end of an 8-hour time period. In comparison to chloroquines 60 % viability, this makes mefloquine approximately twice as effective in the same time period.

The drug with the largest impact on the parasite population was definitively artemisinin with a substantial loss of 85 % of parasite viability over the 8-hour time period in relation to the control.

The drugs selected for use in the assay include some of those most commonly used to combat both the acute and chronic forms of malaria and hence were expected to have respectable efficacy, as evidenced by the nanomolar IC_{50} values obtained in the pLDH assays (not including doxycycline). This was therefore not the purpose of the assay developed in this study.. The focus of this study was rather to develop a novel assay to determine the rate of drug action and hence further prioritize compounds previously found to have antimalarial drug efficacy. The PI and calcein-AM fluorescent vitality probes proved to not only be good indicators of dead and viable parasites respectively, but were able to co-stain without losing any of their vitality marker properties. The drugs used in the study had no noticeable

interaction or adverse effects on the probes themselves and hence the time point study could be concluded to be a valuable insight into the individual rates of action.

2.4 – Discussion

Until recently the rate of drug action has been measured by light microscopic examination of parasite morphology using blood slides of drug treated parasite cultures at regular time intervals. This technique is tedious and, most importantly, subject to interpretation with regards to distinguishing between viable and comprised parasite cells, thus making it impossible to objectively quantitate the rate of drug action. The recently developed viability-based assay by Sanz et al., (2012) determines the rate of action of antimalarials in culture by quantifying the number of parasites capable of recovering from distinct periods of drug exposure. This method is extremely time consuming with the total assay process requiring weeks of culture monitoring post drug exposure to study any re-growth. It is therefore essential to add additional novel assay techniques to the malaria drug discovery arsenal that deal with the specific malarial drug challenges described. Chapter 2 of this study contributed to the development of such a bioassay that is semi-quantitative and capable of rapidly determining the kinetics of drug action in culture.

2.4.1 - Mammalian Cell Staining

The fluorescent vitality probes selected for this study are predominantly marketed for mammalian cell use and although shown to be able to stain their respective cellular elements in other organisms including bacteria (Vesey *et al.,* 1994), and protozoa (Mossallam, 2010) they are widely utilized in mammalian cell studies.Although the probes selected have been used in *Plasmodium* parasites, this has been as a screening tool to determine drug efficacy, i.e. to enumerate the amount of parasites remaining after drug treatment, as opposed to determining the rate of drug action. The studies also often make use of flow cytometry

instead of fluorescence microscopy. In order to understand their innate staining patterns and gain familiarity with their use,it was therefore opted to first assess their staining patterns in mammalian cells prior to their use in *P. falciparum* malaria parasites. Gramicidin S was selected for its cited rapid cytotoxic action in Hela cells in the low µM range using trypan blue and fluorescence microscopy (Rautenbach *et al.,* 2007) and has been shown to lead to lysis in Hela cells within 30 min (H.Hoppe, unpublished).

Drug treated cells showed a significant increase in DAPI staining and a distinct decrease in fluorescent calcein. DAPI is consistently used in literature as a dead cell indicator and often only fixed (via methanol for example) cells will exhibit DAPI fluorescence (Prinsloo *et al.,* 2009). The staining pattern of the cleaved calcein was observed to have no particular subcellular localization and instead encompassed the entirety of the HeLa cell. This would suggest the concentration was unnecessarily high such that intracellular esterases were becoming oversaturated and allowing for the calcein to cross organelle membranes as previously observed by Tenopoulou *et al*., (2007). This was dismissed after significantly lower concentrations either failed to produce fluorescence or the fluorescence signal was weak but still retained by the whole cell. Hence it was determined that the calcein-AM probe acquired functioned as would be expected in mammalian HeLa cells.

Propidium iodide has been used extensively to characterise a reduction in cellular vitality and ultimate cellular degradation.It was observed that only cells that could be classified as ‘dead’ were capable of retaining the probe and as such drug treated cells showed an increase in DAPI staining and a co-localized increase in PI staining. If a cell was unhealthy but appeared to be morphologically still intact and hencehad not reached a sufficiently low vitality threshold then the probe was either not able to enter the cell or the subsequent fluorescent signal was low. In contrast, DAPI appeared to be retained in cells that were both

compromised as well as dead. This is synergistic with a study conducted in papillary thyroid carcinoma cells whereby PI was used to probe for dead cells and DAPI used to further isolate these cells by observing the staining patterns in compromised nuclei (Infanger *et al.,* 2006). The staining patterns observed by the Infanger group (2006) closely resemble those observed in this study and therefore it satisfied the study of acting as a suitable viability probe in mammalian HeLa cells.

In the TMRE samples drug treated cells showed a minor increase in DAPI staining whereby no marked difference was observed in the TMRE test samples. The optimization of the staining patterns focusing on time and concentration combinations of TMRE did not prove fruitful. Staining appeared to occur irrespective of drug treatment and fluorescent intensity did not appear to be affected by cellular vitality. Another ancillary observation was that DAPI staining was unreliable and did not appear to stain all cells that would be considered unhealthy as was seen with the other probes. This could be due to the large scale sequestration of the TMRE stain which potentially interfered and inhibited DAPI access to the nucleus. An important note is that gramicidin S is lytic, cationic and amphipatic and the scenario exists whereby it may have bound to TMRE and hence limited the drug activity against the cellular and mitochondrial membranes. If TMRE could be optimized to localize to the mitochondria as previous diagnostic studies have confirmed (Loew *et al.,* 1993; Collins *et al.,* 2002) then this could allow for less dual probe interaction. It was therefore decided that TMRE would not be suitable as an indication of cellular vitality in this study but would however be attempted in *P. falciparum* parasites to compare staining patterns to mammalian cells.

Of the four vitality probes chosen for the purposes of this study, the most promising prior to experimentation was hydroethidine. Extensive studies have been conducted on the stain as a

vitality probe including work done in mammalian cells (Zielonka *et al.,* 2005) and parasites (Elloso *et al.,* 1994;Cruz *et al.,* 2012). It was unfortunate when the staining patterns could not be optimized sufficiently to provide a reliable vitality indicator in HeLa cells. Drug treated cells showed a large scale DAPI fluorescence as well as co-localization with hydroethidine. Untreated cells showed minor DAPI fluorescence coupled with high fluorescence intensity in hydroethidine positive cells.The impressive sequestration of fluorescence in drug treated cells was unexpected as previous studies have shown that the accumulation of the probe depends heavily on the metabolic state of the cell, with compromised cells exhibiting a significantly reduced amount of fluorescence (Bucana *et al.,* 1986). The co-localization with DAPI would support the conclusion that these cells were indeed membrane compromised but still retained the hydroethidine stain, a result not found in other studies. There exists the potential that gramicidin S treatment somehow leads to compromised cells that retain the ability to be stained with both hydroethidine and TMRE and as such does not conform with expected staining patterns, although further investigation with alternative cytotoxic compounds is required for any confirmation. As was the case with TMRE, hydroethidine was temporarily discarded as a suitable vitality indicator, but would still be pursued in parasites to compare staining patterns.

2.4.2 - Fluorescent Vitality Probe Staining and Optimization in *P. falciparum.*

Calcein-AM staining in *P. falciparum* parasites showed fluorescent coverage of the parasite matrix and absence from the parasite food vacuole. Fluorescent signals observed in healthy trophozoites were found to be of sufficient quality at 1 µg/mL, a five times decrease from that required for mammalian HeLa cells in this study. This could be attributed simply to the size of the parasite being significantly smaller than the HeLa cell and hence requiring lower concentrations of probe, or a higher activity of esterases required to cleave the calcein-AM

precursor to its fluorescent cell-retained product in parasites. Schizont staining followed similar patterns to trophozoite although signal intensity was decreased in comparison. It is known that although large scale metabolism is seen in the trophozoite blood stage, it is reduced in the schizont phase and instead DNA synthesis is upregulated (Fu *et al.,* 2010). It could therefore be hypothesized that the esterases required for cleavage of the acetoxymethyl ester of calcein into its fluorescent counterpart are down regulated in schizonts and hence lower fluorescent signals are observed.

In addition the calcein staining was not limited to the parasite, but instead stained the cytoplasm of infected and uninfected RBC's. This would introduce subjectivity based on the user who may mistakenly classify a RBC as a healthy parasite. Furthermore, any fluorescent measurements using a flow cytometer or plate reader would deduce a signal from a RBC as a parasite fluorescent signal. It was decided to therefore include an additional saponin lysis step which was found to be effective at lysing the RBC membranes and removing the calcein found in the RBC cytoplasm, while leaving intact, easily distinguishable fluorescent parasites. Although there appeared to remain RBC 'ghosts' (RBC membrane remnants surrounding intact parasites), their fluorescence intensity was significantly lower than that of the parasite cell and hence can be phased out using a baseline on a plate reader or flow cytometer. Although the saponin lysis treatment adds an additional step to the assay and can be seen as detrimental to the goal of producing a high throughput procedure, it is essential for accuracy when extrapolating to a quantitative assay using flow cytometry.

A significantly lower concentration of PI was required for adequate parasite staining (3.3 µg/mL) as opposed to mammalian cell staining (20 µg/mL) with just over a six times lower concentration required. A similar trend was observed when moving from mammalian cell staining to parasites using calcein-AM. These decreases are significant in the sense that they

decrease the cost of a new bioassay on laboratories wanting to establish screening facilities. The fluorescence signals were observed across the parasite and were generally absent from the food vacuole however DAPI staining was less pronounced in relation to PI.Other studies have made use of PI concentrations ranging from 5 µg/mL (Liu *et al.,* 2005) through to the most popular 10 µg/mL (Lopez *et al.,* 2010; Boissiere *et al.,* 2012) and up to a staggering 50 µg/mL (Contreras *et al.,* 2004). We found this to be unnecessary and in combination with a 45 min 100 pM gramicidin D incubation provided sufficient membrane deterioration to allow for ideal PI staining. Gramicidin D was selected as a control drug due to its rapid mode of action as shown by Khan *et al.*, 2012.

Lower concentrations or incubation times and PI staining was erratic often being unable to enter the cell. Higher concentrations or longer incubations with gramidicin D weakened the parasites significantly thereby not allowing for the retention of the stain or excessive cellular damage was observed (as measure by light microscopy). Scanning electron microscopy or transmission electron microscopy could be included in the study to enhance the confirmation of cellular damage as well as compare damage inflicted by the different drugs.

An interesting discovery was that PI appeared to stain the matrix of healthy merozoites whereas calcein was not retained in these RBC-free invasive forms of the parasite. The cells were untreated and had been obtained from normal cultures.The observable strong PI staining and lack of calcein could provide false positives for nonviable parasites.It would have been expected that the merozoite membrane would be permeable to calcein-AM and once across the barrier would result in fluorescent green calcein. However, it can be hypothesized that the cellular machinery and esterases required for calcein-AM cleavage are not ubiquitously expressed. As such calcein-AM is able to cross the membrane of the merozoite but remains uncleaved and is able to freely move back into the extracellular space. Alternatively,

merozoite membrane composition differs from the trophozoite stage parasite in the sense that merozoite membranes contain a thick 20 nm surface coat that encodes various marker proteins required for RBC invasion (Maitland and Molyneux, 2004). This variance in membrane composition may account for PIs' capability to cross viable merozite membranes and provide intracellular fluorescence while it is excluded from viable trophozoite parasites. Conversely, it might provide an alternative explanation for the inability of calcein to accumulate in merozoites.

Marketed as a mitochondrial membrane vitality stain and indicator of cellular degradation, TMRE was expected to be a suitable addition to the candidate probes to measure vitality in malaria parasites. HeLa cell staining showed no specific localization and instead whole-cell, cytoplasmic staining, as well as potentially organelle staining. In contrast, TMRE was shown to have a moderate preference for regions in malaria parasites although it is unclear if this localization is mitochondria specific (morphologically, the staining does not resemble the typical patterns obtained when staining parasites with mitochondrial marker probes, e.g. Mitotracker) and staining patterns were mostly irregular. Studies that made use of the probe for measuring cellular apoptosis in *P. falciparum* have shown less than desirable localization (Mutai and Waitumbi 2010) and instead appear to more closely resemble the HeLa cell staining observed in this study. While attempts were made to optimize the staining patterns in the parasite, focusing on duration and concentration of probe treatment, it was still observed to be able to stain both live and compromised cells in all cases, as was evident in HeLa staining,and it was decided to no longer continue pursuing TMRE as a suitable fluorescent vitality probe.

Although used in various studies and organisms as a distinct indicator of viable cells it was observed that similarly to HeLa cells, there was no decrease in hydroethidine fluorescence

after drug treatment with gramicidin D. This probe had been most extensively used as a vitality marker in parasites based on published results (Krishan, 1975; Elloso *et al*., 1994; Cruz *et al.,* 2012) and hence it was decided to focus an extra amount of time to attempt to optimize the stain to provide valid vitality effects. The concentration of the probe was altered systematically, as well as the incubation temperature although to no resultant change in staining patterns. It has been reported that in the trophozoite life stage of the parasite a decline in fluorescence intensity is observed to accompany a decrease in parasite viability (Chevalley *et al.,* 2010). Although this study found staining patterns that closely resembled those published by the Chevalley group (2010), it was unable to show a change in those patterns after drug treatment. Furthermore, studies conducted on all life stages of the parasite provided the same outcome – high DAPI and hydroethidine fluorescence in both drug treated and viable cells.

The technical data sheet makes note that hydroethidine staining is sufficiently observed at room temperature (Polysciences, Inc. 2000), however, all staining was completed at 37^{o}C and under special gas (5% CO_2 ; 5% O_2 ; 90 % N_2) to replicate procedures followed for the other probes as well as to simulate optimal parasite culture conditions. Therefore, a parasite sample was stained at room temperature alongside a sample stained at 37^{o}C and a marked decrease in fluorescent intensity was observed in the room temperature cells suggesting that the use of a higher temperature in this study is an improvement on existing staining procedures. Additionally, extreme concentrations of hydroethidine(50 µg/mL) have been published (Chevalley *et al.,* 2010) and are unnecessary for effective *P. falciparum* cell staining. Sufficient fluorescence was observed at as little as 1 µg/mL.Further assays were conducted increasing the hydroethidine concentration to 3 µg/mL and 6 µg/mL to assess whether or not the decrease in fluorescence intensity in compromised parasites reported by Chevalley *et al*.,

(2010) and others was hydroethidine concentration dependent. This however had no effect and hence hydroethidine was discarded as a suitable vitality probe for use in this study.

2.4.3 - pLDH Assay to Determine Drug Dose Response Curves and IC_{50} values

Although IC_{50} values for the standard antimalarial drugs used in this study have been reported in numerous studies, it was important to optimise the effective concentrations of the drugs to ensure reproducibility and to control a variety of factors: i) that the drug stocks used in this study were active; ii) that the 3D7 parasite strain used in this study had not altered its susceptibility to the drugs due to extensive sub-culturing; iii) that our routine culturing conditions and procedures don't result in altered drug sensitivity. Reassuringly, the IC_{50} values obtained agreed closely with published results. Artemisinin showed a significant and rapid change between a drug concentration incapable of eliminating parasites and one that provided a near 100 % decrease in parasite vitality which provided a pinpoint IC_{50} of 16 nM which deviates only slightly from the cited literature value of 24 nM (Walcourt *et al.,* 2008).

The IC_{50} value of 12 nM for chloroquine concurred with a cited literature value of 14 nM which was also tested on the 3D7 strain of *P. falciparum.* (Moneriz *et al.,* 2011). It is important to note that the 3D7 strain is a chloroquine sensitive strain of the parasite which will naturally not necessarily provide an accurate indication of chloroquine dose response on wild type malaria parasites. Studies show that chloroquine resistant strains can require a ten times increase in chloroquine concentration required to provide 50 % parasite vitality inhibition as was, for example, witnessed by the Moneriz group when an IC_{50} of 190 nM was calculated for the chloroquine resistant strain *P. falciparum* Dd2 (Moneriz *et al.,* 2011).

Mefloquines dose response curves provided the lowest IC_{50} out of the five compounds under evaluation at 9 nM which correlates well to literature at 12 nM (Wisedpanichkii *et al.,* 2009). As with chloroquine, the Wisedpanichkii group (2009) used the chloroquine sensitive strain

3D7 to conduct their IC_{50} determination and hence can be directly compared with this study. Although mefloquine belongs to the quinine family of drug compounds as chloroquine does it is unconfirmed whether or not they share similar mechanisms of action. Further IC_{50} experiments conducted on the chloroquine resistant strain K1 provided an IC_{50} of 9 nM, a statistically significant relation to the IC_{50} obtained from strain 3D7 which alludes to the possibility of dissimilar mechanisms (Wisedpanichkii *et al.,* 2009).

For doxycycline,although the test IC_{50} compared well with the literature value, 12 µM and 11 µM respectively, these values were determined using the 3D7 strain of *P. falciparum* and can vary with wild type parasites (Fall *et al.*, 2011). A study conducted by Briolant *et al*., (2009) obtained IC_{50} concentrations for doxycycline in 747 isolates from patients divided into three groups in Senegal. Mean IC_{50} concentrations for the groups ranged from 5 µM to 18 µM showing minor variations even in inter-population studies (Briolant *et al.,* 2009).

Although not commonly used as a mainstream antimalarial, methylene blue (MB) provided an IC_{50}comparable to those of the other test compounds. The IC_{50} for MB was 12 nM as opposed to only 2 nM reported in literature (Okombo *et al.,* 2012). The reason for this disparity is not clear. Discontinued in malaria treatment owing to the side effect of turning urine blue, it has since been used extensively to treat methemglobinemia (Okombo *et al.,* 2012). With the burgeoning problem of drug resistance as a real and current threat there has since been increased interest and renewed vigour in studying this compound (Dormoi *et al.,* 2012).Furthermore MB has been proven to be effective in significantly reducing gametocytaemia in studies conducted in Burkina Faso (Coulibaly *et al.,* 2009). It was determined that MB was able to act against both developing and mature gametocytes, thus having the potential to reduce transmission in malaria endemic regions (Coulibaly *et al.,* 2009).

An accurate IC_{50} for the individual compounds was required to determine the concentrations to be used in the rate of action experiments using the vitality probes. Two considerations went into the choice of using a 10 x IC_{50} concentration for subsequent studies. Firstly, a sufficiently high concentration is required that will confidently compromise the viability of all the parasites in the test sample in all instances, taking into account the fluctuation in effective drug concentration that is typically found from one experiment to the next and is a normal consequence of the variables involved in working with live cells. Secondly, the concentration should ideally be sufficiently low for the results to reflect the normal mode of action of the drug in question, and not be a consequence of off-target effects (i.e. the compound inhibiting proteins and pathways unrelated to its primary target at high concentrations).

2.4.4 - Using the Optimized calcein-AM and PI Fluorescent Probe staining procedures with standard antimalarial compounds.

Calcein-AM and PI were selected as promising parasite viability markers for rate of action experiments by using gramicidin D as a model compound known to rapidly affect parasite viability (Khan *et al.*, 2012). The question was whether these probes would be useful in conjunction with the more conventional, standard antimalarial drugs for which the IC_{50} values were determined. In addition, during the optimization stage the probes were used individually. A second question was whether they could be used in combination to simultaneously detect and quantitate viable (calcein positive) and non-viable (PI positive) parasites in the same sample. In the experiment that was thus performed, parasites were incubated in parallel cultures with the standard antimalarials for 8 hours, stained with a combination of calcein-AM and PI and viewed by fluorescence microscopy. Percentage parasite viability could then be calculated from the ratio of viable (calcein positive) and non-

viable (PI positive) parasites. Compounds with a more rapid rate of action would thus be expected to yield comparatively lower percentage parasite viability at the 8 hour time-point.

Strikingly, the calcein-AM and PI stains, used in combination, complemented each other extremely well. With the individual drugs, a decrease in calcein stained parasites was mirrored by a complementary increase in PI staining, and no co-staining was observed. The probes can therefore be used to robustly distinguish and quantify viable/non-viable parasites in cultures treated with conventional antimalarials. Moreover, the 8 hour time-point chosen for this study proved to be very effective for determining the comparative rates of action of the individual drugs. Based on the percentage parasite viability at this time-point, the study drugs can be ranked (as a measure of remaining parasite population after drug treatment) as gramicidin D (2% remaining viability) > artemisinin (15%) > mefloquine (33%) > chloroquine (60%) > doxycycline (86%), which agrees very well with published studies (e.g. Khan *et al.,* 2012) and their known properties. This strongly suggests that the assay, even in its current form, can be used to rank the rate of action of experimental antimalarial compounds *vis-à-vis* each other and standards.

The commonly prescribed semi-synthetic doxycycline is principally advocated for its potent antibacterial properties and only secondarily as an antimalarial. Therefore it is not unexpected that its bactericidal mechanism of action may not correlate with that of the antimalarial efficacy. However, it has been established that the mechanism observed in bacteria is due to the inhibition of protein synthesis at the ribosome whereby the compound binds to the 30S small ribosome subunit preventing the further attachment of the tRNA (Majewski, 2014). In *P. falciparum* doxycycline has been shown to interact with and disrupt nucleotide synthesis (Yeo *et al.,* 1998), as well as inhibit mitochondrial protein synthesis (Blum *et al.,*1984).

A later study showed an effect of doxycycline on the parasite apicoplast albeit the effect was late onset due to the slow action of the drug (Dahl *et al.,* 2006). Parasites were treated with doxycycline and assessed after 12 hours, after which parasite apicoplasts appeared morphologically intact and were capable of replicating their genomes for segregation into developing merozoites (Dahl *et al.,* 2006). It was observed that instead of having a direct impact on the apicoplast that doxycycline disrupted the expression of apicoplast genes encoded by the nuclear genome. Hence doxycycline would not show an effect on existing apicoplast function, but instead apicoplasts inherited by daughter merozoites of doxyxcycline-treated parasites would contain insufficient levels of apicoplast-encoded proteins required for normal function (Dahl *et al.,* 2006). This loss in apicoplast function would ultimately result in parasite cell death, albeit in the subsequent parasite life cycle post doxycycline treatment.

The important factor to note is that doxycycline treated parasites appeared morphologically intact until only late in the second 48-hour life cycle of the treated parasites (Dahl *et al.,* 2006). This was still evident even if the drugs were removed after the initial cycle post-treatment. This would explain and confirm the significantly slow rate of action observed in doxycycline treated parasites in this study and suggest that there should be little to no effect on the parasite population over the course of an 8-hour incubation period.

Chloroquine, a member of the quinolones, was once the gold standard of malaria treatment until the rapid emergence of chloroquine-resistant parasite strains. (Meshnick and Dobson, 2001). It is thought to disrupt the parasite machinery involved in haem detoxification and hence result in a build-up of toxic haem and resultant cell death (Chinappi *et al.,* 2010). The malaria parasite relies on the metabolism of the RBC cytoplasm, which is made almost exclusively of haemoglobin, to provide the necessary amino acids for protein synthesis. As

such the parasite consumes up to 80 % of the RBC's inner cytoplasm at the peak of its metabolism (Francis *et al.,* 1997). Haem is toxic to the malaria parasite and has been shown to cause complete parasite cell lysis, therefore haem detoxification is essential during parasite metabolism (Fitch *et al.,* 1982). This detoxification involves a polymerization of the haem group in a process resulting in the chemically inert haemozoin contained within the parasite food vacuole (Francis *et al.,* 1997).

Chloroquine is proposed to disrupt the sequestration process resulting in a build-up of toxic haem and resultant cell death (Chinappi *et al.,* 2010). This is achieved by chloroquine accumulating in the parasite food vacuole where it is trapped due its basic properties. The drug then binds to the haem as it enters the vacuole and prevents the conversion to haemozoin. As such the build-up of the toxic haem results in eventual parasite cell lysis due to its ability to disrupt the permeabilization of the parasite cell membrane (Fitch *et al.,* 1982). Alternative studies have shown chloroquine to interact with and inhibit the parasites native catalase and peroxidase enzymes and hence result in an increase in reactive oxygen species and ensuing DNA degradation (Bandyopadhyay *et al.,* 1999).

Due to widespread resistance it has since become less used in isolation and instead is combined with other drugs to create 'cocktails' to combat resistant strains. This study showed chloroquine to be a relatively slow acting drug in comparison to controls and other test compounds. With a mean parasite viability of 60 % this shows that after 8 hours just over half the parasite population was still viable enough to metabolise and retain calcein fluorescence and exclude PI, suggesting that the haem accumulation due to chloroquine action has a relatively slow cytotoxic effect compared, for example, to the actions of artemisinin and mefloquine. This conclusion correlated with findings by Khan *et al.* (2012) in which chloroquine was found to exhibit a slow rate of antimalarial action using an ATP and

luciferase based bioassays to measure the rate of drug action against *P. falciparum.* This study showed the onset of parasite cell death only at later time intervals and is further supported by Ginsburg *et al.* (1998) who observed late onset of parasite cell death after haem levels were critical.

As a member of the quinoline family it would perhaps be expected that mefloquine would provide a similar mechanism and similarkilling rate to chloroquine in malaria parasites. This has been disproven in various studies that have shown differing rates and concentrations of the two compounds required for parasite inhibition. On the other hand, combination therapies encompassing both drugs appear to provide a more effective outcome than either of the drugs alone which suggests a synergistic relationship (Sowunmi and Oduola, 1997). Initially prescribed in Thailand as a combination therapy (mefloquine, sulfadoxine and pyrimethamine) in 1987 it has since been adapted for use in ACT (artemisinin combination) therapies in order to combat the increasing number of resistant malarial strains (Na-Bangchang *et al.,* 2007).

Mefloquine has been the topic of vastly debated mechanisms of action of which the opinions vary amongthe targetting of haem (similarly too chloroquine) and phospholipid metabolism(Fitch, 2004)as well as been shown to bind to haem directly (Dorn *et al.,* 1998). It has been suggested that it may inhibit the decomposition of haem and in turn enhance the binding of haem to parasite membranes although this was to a lesser extent in relation to chloroquine (Loria *et al.,* 1999). Extensive studies have exhibited a disruption effect of mefloquine on the polymerization process of haem to haemozoin (Sullivan *et al.,* 1998; Combrinck *et al.,* 2013). On the other hand there is evidence to suggest a contrasting mechanism from that of chloroquine. It has been shown that mefloquine has a strong interaction in the parasites endocytosis pathways whereby after treatment with mefloquine

there was a reduction in the percentage of parasites with multiple transport vesicles (Hoppe *et al.,* 2004). The study noted that this mode of inhibition was negligibly seen in chloroquine treated parasites (Hoppe *et al.,* 2004).

It was found that in this study that mefloquine was more rapidly acting in comparison to chloroquine with only 33 % of parasites remaining viable at the end of an 8-hour time period – this in comparison to chloroquines 60 % viability makes mefloquine almost twice as effective in the same time period. This further suggests a differential mechanism between the two drugs and a higher susceptibility of the 3D7 strain of the parasite to mefloquine.

The drug that showed the largest impact on the parasite population was definitively artemisinin with a substantial loss of 85 % of parasite viability over the 8-hour time period in relation to the untreated control. Although it is known that artemisinin and its derivatives have significantly shorterhalf-livescompared to other antimalarials, it is difficult to ascertain the exact critical point of the parasite population collapse. The mechanism of action of artemisinin has been debated for as long as its structure has been known. It was in fact the structure of the compound that brought about the first clue to its mechanism as it was shown that the endoperoxide bridge was essential for its activity (Brossi *et al.,* 1988). Reactive oxygen species arise from a variety of sources, one being peroxides (such as superoxide) suggesting that free radicals might well form the basis of the mode of action (Krishna *et al.,* 2004). This would pertain to the iron of digested haem reducing a peroxide bond in the drug resulting in the generation and accumulation of reactive oxygen species leading to parasite cell death (Cumming *et al.,* 1997). Alternatively it has been suggested that the drug may target a particular protein or enzyme. Work in this area has shown artemisinin to inhibit the falcipain protein family - cysteine proteases that promote heme degradation (Pandey *et al.,* 1999). Other work has shown artemisinin to accumulate within parasite lipids causing

membrane damage (Hartwig *et al.*, 2009) as well as be a disruptor to the electron transport chain and host cell mitochondria (Li *et al.*, 2005).

It has been shown that the active metabolite has an elimination half-life of approximately one hour. However, studies have not shown a measure of overall malaria parasite viability at this same time point (Krishna *et al.*, 2004). Sanz and company assessed the *in vitro* killing rate of artemisinin albeit their focus was on the parasites ability to recovery post-drug treatment and hence their first measurement of any reduction in vitality was at 24-hours and showed almost complete population downfall (Sanz *et al.*, 2012). Sullivan observed the effect on the parasite after artemisinin treatment at specific points during the life cycle and noted a hypersensitivity of parasites less than eight hours old to the drug (Sullivan, 2013). Furthermore, it was demonstrated that short pulses of between one and six hour doses of artemisinin were effective in detecting significant reductions in viability. This is the most rapid rate of action for any antimalarial proposed in literature and correlates well with the killing rate observed in this study (Sullivan, 2013).

2.5 – Conclusion

The probes selected for use in this study (calcein-AM, PI, TMRE, hydroethidine) are more widely utilized in mammalian cells, although shown to be able to stain their respective cellular elements in other organisms including bacteria and protozoa. In order to gain familiarity with the characteristics of the probes (e.g. optimal concentration, incubation times, staining intensity, ability to distinguish viable vs. non-viable cells) it was therefore practical to assess their staining patterns in mammalian cells prior to their use in *P. falciparum* malaria parasites. Cultured mammalian cells were treated with a standard (cytotoxic peptide) lethal compound (gramicidin S), incubated with the vitality probes and staining patterns assessed by fluorescence microscopy. Calcein-AM (staining the cytoplasm of viable cells) and PI

(staining the nuclei of nonviable cells) provided the most suitable staining patterns in relation to the other test probes i.e. TMRE and hydroethidine. The latter two probes failed to distinguish live and compromised cells. A significant breakthrough was to confirm that this staining behaviour of the former two vitality probes extended to *P. falciparum* parasites as well. After further optimization of incubation conditions, calcein-AM and PI staining were able to robustly and reproducibly distinguish between viable/compromised parasites after drug treatment.

Before the fluorescent vitality assay could be conducted on the standard antimalarial drug standards it was necessary to test their efficacy (i.e. the concentrations at which they're effective) against the laboratory strain of *P. falciparum* 3D7 parasites. Drug concentrations resulting in 50 % parasite cytotoxicity were determined using the lactate dehydrogenase (pLDH) cytotoxicity assay and used to guide the concentrations employed in the vitality probe bioassay.

Further development of the bioassay involved staining parasites with a combinationof the calcein-AM and PI probes after incubation with the respective drugs in the panelfor eight hours. Changesin parasite staining patterns were semi-quantitatively analysedby fluorescence microscopy using ImageJ v1.49 and the Cell Counter plugin i.e. counting the ratio of viable (calcein positive/PI negative) vs. non-viable (calcein negative/PI positive) parasites in the treated cultures. It was observed that a decrease in calcein and increase in PI fluorescence (i.e. compromised parasite viability)was progressively seen moving from the expected (based on literature reports) slowest acting drug (doxycycline) through to the most rapid acting (artemisinin). These results strongly suggested that the assay was indeed capable of ranking antimalarial compounds based on their individual rates of action.

The next step in the study was to make use of additional techniques to improve the quantification of the novel bioassay which was done using flow cytometry. Furthermore, to produce a more "user-friendly" assay for wider utilzation, we explored the possibility of using a multiwell fluorimeter to quantitate calcein and PI fluorescence in drug treated parasite populations. Additionally comparisons were made between the newly generated rate of action assays (both fluorescence microscopy and flow cytometry read outs) to that of a modified parasite reduction ratio (PRR) assay (Sanz *et al.*, 2012) as well as the ATP assay (Khan *et al.*, 2012).

Chapter 3 - Increasing the Throughput and Quantification of the Fluorescent Probe Assay and Comparisons to Current Assays

3.1 - Introduction

Fluorescence based assays that make use of DNA intercalating dyes have long since been used to measure malaria growth susceptibility to drug compounds. They include the PicoGreen, YOYO-1 and, arguably the most common, SYBR green assays (Bennet *et al.*, 2004; Corbett *et al.*, 2004; Banieckie *et al.*, 2007). These assays are highly sensitive and allow for rapid quantification and assessment of parasite drug pressure *in vitro* while they fail to account for the rate of individual drug action. Prior to the breakthrough PRR assay developed by Sanz and colleagues in 2012, the rate of drug action had been measured using Giemsa-stained blood slides of drug treated parasite *in vitro* at regular time intervals.This technique is tedious and subject to interpretation with regards to distinguishing between viable and comprised parasite cells. The Parasite Reduction Ratio (PRR) assay provided a more adept means of assessing the *in vitro* killing rates of antimalarial drugs and while the assay is essentially definitive it is time consuming and limited in throughput. Recent developments in attempts to explore ways of measuring *in vitro* drug killing rates saw Khan and colleagues (2012) utilize native parasite ATP levels as an indication of drug stress by quantifying parasite numbers post drug treatment allowing suggestions to be made on the rate of antimalarial drug action. This was based on the luciferin-luciferase system and culminated in an increase in ATP levels in the parasite post drug treatment (Khan *et al.*, 2012).

Chapter 2 of this study proposed and provided evidence for the development of a novel bioassay in which the rate of drug action on malaria parasites was assessed in a semi-quantitative manner using fluorescence microscopy. This chapter aimed to improve on this

novel assay by i) generating a fully quantifiable bioassay and ii) increasing the output to medium-high throughput using flow cytometry. This improved fluorescence-based assay was then compared the PRR and ATP assays described. Furthermore, the feasibility of adapting the fluorescence based assay for use in a multiwell fluorimeter was assessed.

3.2 - Materials and Methods

3.2.1 - Quantifying the FluorescenceAssay using Flow Cytometry.

Initial stages of the assay were carried out as per section 2.2.7 of Chapter 2 albeit without the transferral of stained test samples to microscope slides. At this point each treated sample was instead transferred to a 5 mL flow cytometry round bottom test tube before analysis using a FACS Aria II flow cytometer. The calcein-AM (FITC) was excited at 488 nm and emission recorded in the 530/30 filter channel, while the 622 nm laser was used to excite PI and emission recorded in the 660/20 filter channel. The UV laser was used to excite Hoescht 33342 (Hoescht) at 355 nm. A total of 100 000 events were recorded for each sample and the data was analysed using FlowJo software (Tree Star Inc.). Gates were established to isolate the parasite population from residual RBC's as well as to further isolate trophozoite stage parasites for other asexual life stages. Dot plots were generated depicting parasite cell counts as a measure of fluorescent events – PI positive indicating dead cells and calcein positive indicating live cells.

3.2.2 - Fluorescent Probe Filter Tube Assay

P. falciparum 3D7 parasites were cultured under sterile conditions as described (Chapter 2, 2.2.2). A 10 % trophozoite stage culture was used to prepare final parasite suspensions of 5 % haematocrit and 5 % parasitaemia. Test samples were incubated in their respective drug compounds for 8 hours at 10x their IC_{50} values as per the following profile – artemisinin: 160

nM, doxycycline: 120 µM, chloroquine: 125 nM, mefloquine: 95 nM and methylene blue: 115 nM. All samples were gassed with a special gas mixture (5% CO_2 ; 5% O_2 ; 90 % N_2) before being placed in a 37^{o}C incubator for the duration of the incubation period. To simulate a collapsed parasite population as a negative control, one suspension was incubated with 100 pM of gramicidin D for 1 hour. The gramicidin D treated sample was treated with drug at the 7 hour mark to synchronise with the test samples. An additional control sample was generated by simulating optimum culture conditions in a sample containing parasitized RBC's in CM and no drug treatment, as well as two background control samples containing unparasitized RBC's and parasitized RBC's that were left unstained. After the incubation the drug treated samples were washed in PBS and all but the 'unstained' sample was treated with 2 µM calcein-AM for 30 min after which they were washed twice in PBS. The RBC's were lysed with a mixture of 0.3 % saponin and 0.5 % BSA from where the lysates were transferred to a Spin-X HPLC micro centrifuge filter tube (0.45 µm nylon filter). The lysates were allowed to settle for 5 min after which the filter tube was spun down at 1050 *g* for 3 min and the filtrate discarded. A mixture of 0.5 % Triton-X in PBS was used to lyse the remaining parasites after which they were again spun down 1050 *g* for 3 min. Each respective final lysate was transferred to the well of a 96-well plate and the fluorescence of each well was measured in a multi-well plate reader to measure remaining parasite-associated calcein.

3.2.3 - ATP Assay

Based on the assay developed by Khan *et al.*, 2012 and adapted in this study, *P. falciparum* 3D7 parasites were cultured under sterile conditions as described (Chapter 2, 2.2.2). A 10 % trophozoite stage culture was used to prepare final parasite suspensions of 5 % haematocrit and 5 % parasitaemia. Test samples were incubated in their respective drug compounds for 8 hours at 10x their IC_{50}'s as per the following profile – artemisinin: 160 nM, doxycycline: 120

µM, chloroquine: 125 nM, mefloquine: 95 nM and methylene blue: 115 nM. All samples were gassed with a special gas mixture (5% CO_2 ; 5% O_2 ; 90 % N_2) before being placed in a 37^{o}C incubator for the duration of the incubation period. To simulate a collapsed parasite population as a negative control, one suspension was incubated with 100 pM of gramicidin D for 1 hour. The gramicidin D treated sample was treated with drug at seven hours to synchronise with the test samples. An additional control sample was generated by simulating optimum culture conditions in a sample containing parasitized RBC's in CM and no drug treatment. After the incubation the drug treated samples were washed in PBS and the cells were lysed with a mixture of 0.3 % saponin and 0.5 % BSA from where the lysates were transferred to a Spin-X HPLC micro centrifuge filter tube (0.45 µm nylon filter. The lysates were allowed to settle for 5min after which the filter tube was spun down at 1050 *g* for 3 min and the filtrate discarded. An additional wash step with PBS conducted after which the filters were placed in a fresh epps and equal volume of PBS and CellTiter-Glo reagent (Appendix 11) was added to each filter. The filter tubes were again spun down at 1050 *g* for 3 min and the filtrate transferred to a white-walled, clear bottom 96-well plate and the luminescence of each well was measured in a multi-well plate reader to measure parasite-associated ATP.

3.2.4 - Parasite Reduction Ratio (PRR) Assay

P. falciparum 3D7 parasites were cultured under sterile conditions as described (Chapter 2, 2.2.2). A parasite cultureof between 2-5 % parasitaemia containing healthy, trophozoite stage parasites was transferred to 24-well plates. Control samples included a negative control of unparastized RBC's and a positive control of healthy, untreated, parasitzed RBC's. Test samples were treated with 10 x IC_{50} of the respective compound and the plates were transferred to an airtight container and gassed with a special gas mixture (5% CO_2 ; 5% O_2 ; 90 % N_2) before being placed in a 37^{o}C incubator. After an 8 hour incubation period the plate

was removed and the cells were washed to remove the test compounds. The parasite pellets and respective control pellets were placed in fresh CM and placed in 24 well plates after which they were returned to the air tight container, gassed and allowed to incubate at 37^{o}C for an additional 40 hours. Post incubation a pLDH assay was conducted on each sample to assess parasite viability as a factor of individual drug efficacy over an 8 hour time period.

3.3 – Results

3.3.1 - Quantifying the Fluorescence Assay using Flow Cytometry

The novel fluorescence probe assay described in Chapter 2 provided sufficient evidence that it was able to semi-quantitatively distinguish and separate a panel of antimalarial compounds based on their rates of action. It was considerably less time consuming than the modified PRR assay (requiring only 8 hours of drug incubation prior to analysis as opposed to the 48 hours required by the PRR assay). It was therefore decided to further develop the assay to increase throughput and develop a fully quantifiable rate of action bioassay using flow cytometry and the same fluorescent vitality probes used in the microscopy study – calcein-AM and PI.

The fluorescence assay described in Chapter 2 (2.2.7) was repeated albeit each drug treated and stained parasite sample as well as any control samples was transferred to a 5 mL flow cytometry round bottom test tube before analysis using a FACS Aria II flow cytometer. A total of 100 000 events were recorded for each sample and the data was analysed using FlowJo software (Tree Star Inc.).

It was necessary to ensure the parasites alone could be distinguished from the RBC's such that they could be isolated and any analysis and conclusions made on cellular viability would be a direct reflection of the parasites themselves. Infected RBC's (iRBC's) were stained with

the nucleic acid probe Hoechst and were controlled against unstained iRBC's. Briefly a parasite culture of between 5-10 % parasitaemia was pelleted and 40 µL removed and split between two Eppendorf tubes containing 200 µL of complete media each. One sample was treated with 1 µg\mL Hoechst for 10 min prior to analysis using a FACS Aria II flow cytometer. Hoechst was used as it is not dependent on cellular vitality and hence is able to stain any nucleic acid present.

Upon the inclusion of Hoechst a large increase in DNA stained, Hoechst positive cells emerge from the population as per the gating in the Figure 21.

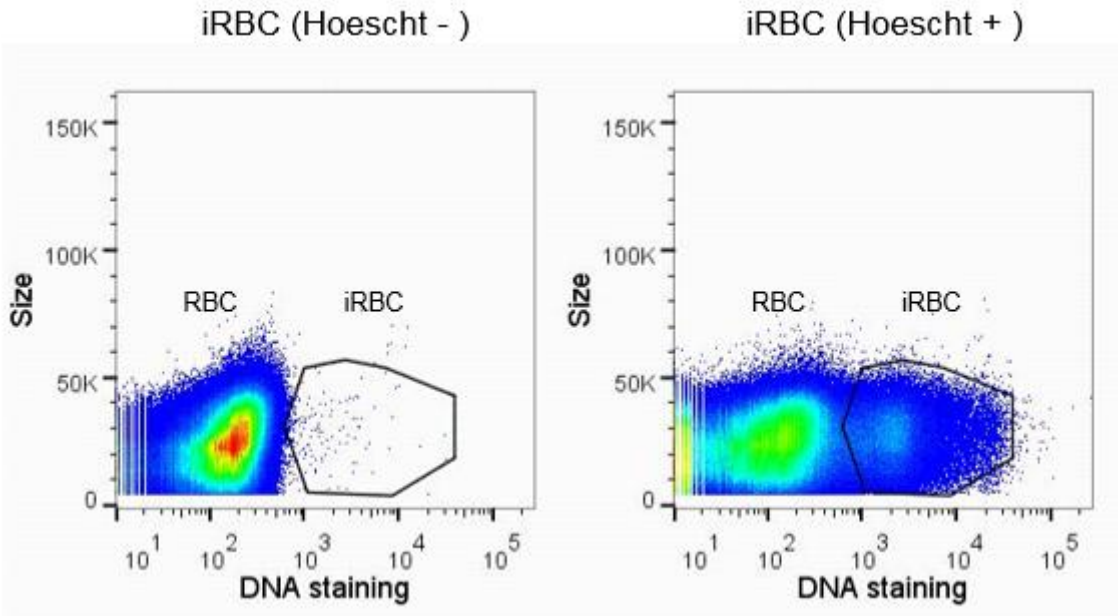

Figure 21: Flow cytometric histogram showing gating on total RBC population such that Hoechst positive cells can be isolated and are indicative of iRBC's analysed using FlowJo software (Tree Star Inc.).

The nucleic acid dye, Hoechst, proved to be an effective probe in dividing the total cellular population into infected and uninfected cells. This was critical if viable cell counts were going to be exclusively determined using the parasite infected population. It was subsequently necessary to assess the vitality probes detection ability in the flow cytometer. It was crucial to ensure they were still able to distinguish between live and dead cell

populations as previously recorded with fluorescence microscopy. The gating determined in Figure 21 was applied in all successive cytometry experiments to immediately remove residual uninfected RBC's.

The assay was again repeated with the addition of a treatment of 100 pM of gramicidin D for 45 min to a sample that was subsequently stained with PI (3.3 µg/mL) and analysed in a FACS Aria II flow cytometer in relation to untreated control and unstained control samples respectively (Figure 22 A). The process was repeated and PI was replaced with calcein-AM (5 µg/mL) and included the same controls (Figure 22 B).

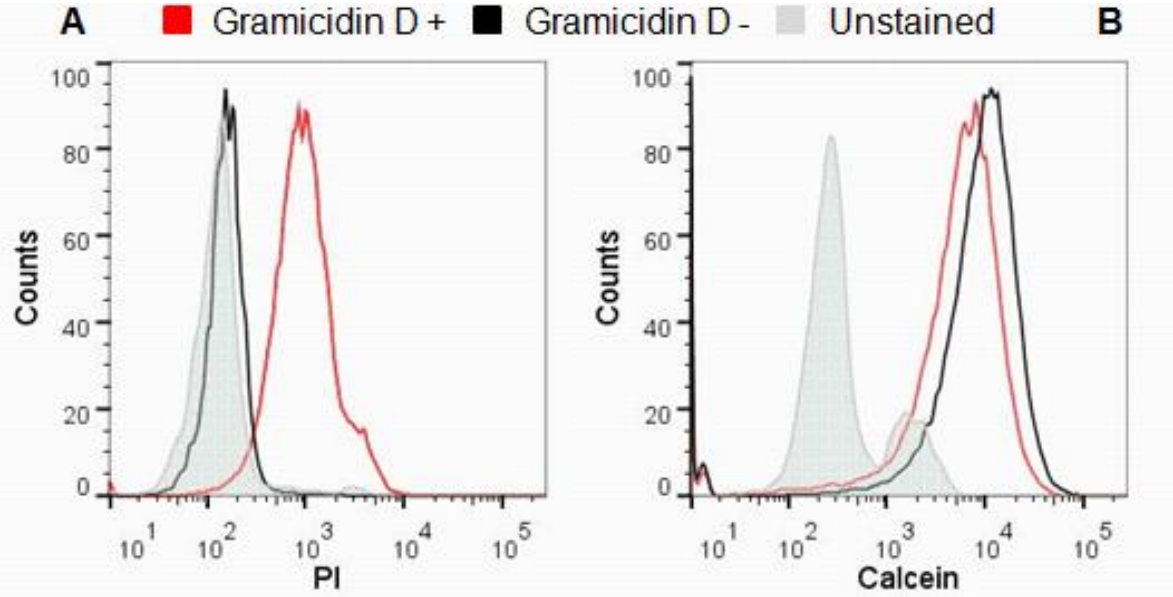

Figure 22: Flow cytometry histograms of gramicidin D (100 pM) treated iRBC's stained with PI (3.3 µg/mL) (A) and gramicidin D (100 pM) treated iRBC's stained with calcein-AM (5 µg/mL) (B). Both panels are in relation to unstained and untreated control cultures and staining is exclusive for the iRBC population. X-axis is representative of PI/Calcein fluorescence intensity.

The unstained control culture allowed for baseline fluorescence and corrected for any auto fluorescence. Gramicidin D positive parasites in panel A show a significant increase in PI staining in relation to the gramicidin D negative and unstained controls. In contrast calcein-AM positive cells (panel B) show little difference between gramicidin D treated and

untreated populations. This can be attributed to the fact that it was observed via fluorescence microscopy that calcein-AM is able to stain the RBC matrix (Chapter 2: Figure 14) and hence provide false positives for healthy parasite cells. It was thus necessary to remove the parasite from the RBC using saponin prior to making any assessments on live and dead parasite populations.

A first step was to determine whether it is possible to detect and quantify individual parasites in saponin-lysed cultures. In this regard parasite populations were stained with Hoechst analysed for Hoechst fluorescence (Figure 23). The ability of Hoechst to only stain nucleic acids would isolate the parasites from the residual uninfected RBC's and RBC debris (ghosts) that survive the saponin treatment. This is due to the fact that RBC's lack any form of native nucleic acid.

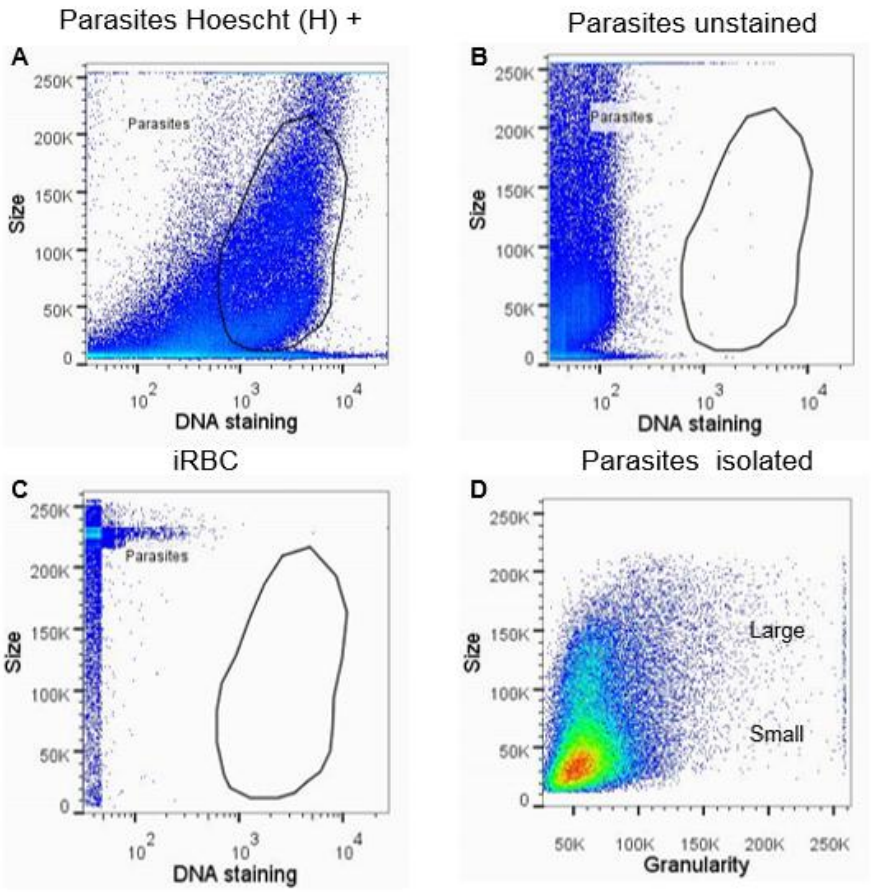

Figure 23: Rationale behind the gates selected to further isolate the parasite population from any remaining RBCs or RBC debris after saponin lysis. A – Parasites isolated according to high Hoechst positive signals. B – Unstained parasites confirming no population present in the absence of the DNA stain. C – iRBC's serves to depict a lack of RBC signals in the selected gate. D – Size and granularity of the isolated parasite population.

Parasites were identified based on Hoechst staining on the pseudo colour plot of size versus DNA staining intensity. Given that RBC's lack nuclei and hence DNA, the only observable fluorescence was representative of the parasites. The gate observed in Figure 23 surrounding the parasite population (the rough oval seen in all the panels) was established using the stained and control samples, such that it sequesters signals that are high for DNA and excludes any others. The parasite population that were solely Hoechst stained (Figure 23 A) indicated where the necessary gate should be placed to isolate signals with high DNA staining. The unstained parasites depict an absence of the parasite detection in the absence of

Hoechst (Figure 23 B) as well as no overlap of RBC's into the gate (Figure 23 C). Finally the parasites were further isolated to show the individual size and granularity scatter across the population (Figure23 D) and resulted in two emerging sub-populations – attributed to a smaller sized population with lower granularity relating to early trophozoites and a larger, more granular population of mature trophozoites.

With the parasite population identified it was then mandatory to assess the impact on individual parasite populations as a result of model drug treatment. Hence parasites were treated with artemisinin, chloroquine, doxycycline, methylene blue and mefloquine and analysed at three time points to gauge the change in staining patterns between PI and calcein-AM. A general trend of an increase in PI staining can be observed with the time progression (Figure 24).

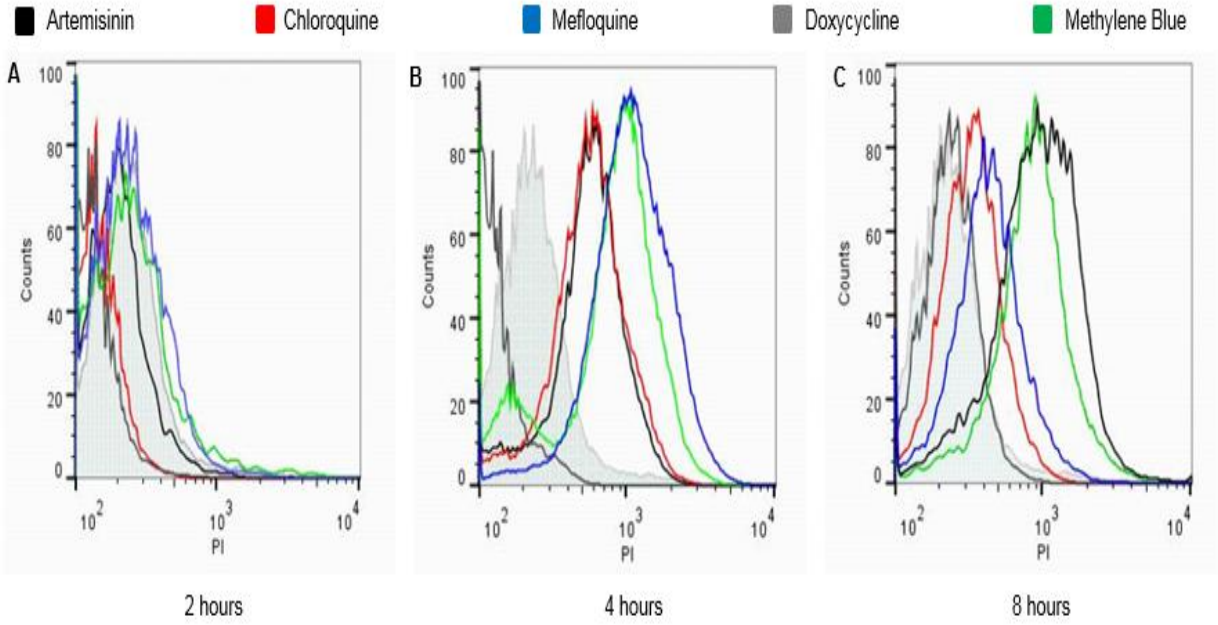

Figure 24: Flow cytometry histogram comparison of the model antimalarial drugs and their respective rates of action as a measure of PI positive events after being treated for 2 hours (A), 4 hours (B) and 8 hours (C) with doxycycline (100 µM), chloroquine (120 nM), mefloquine (90 nM) artemisinin (150 nM) and methylene blue (110 nM) respectively. The shaded peak is representative of unstained parasites. Post treatment the samples were stained with PI (3.3 µg/mL) and analysed using FlowJo software (Tree Star Inc.).

At the 2 hour time point there is very little change in the PI staining pattern between any of the drug treated samples in relation to each other, as well as in relation to the shaded background control region. Variations begin to emerge as early as 4 hours where mefloquine and MB show the largest increases in PI signal. Chloroquine and artemisinin show an almost identical staining pattern with a large increase in signal in relation to the untreated parasites while the slowest acting drug, doxycycline, does not exhibit any PI fluorescence. At the 8 hour interval there is a significant increase in the PI signal received from the artemisinin treated sample as expected from the quickest acting drug. This is closely followed by methylene blue which along with a low literature IC_{50} value of 3 nM and often championed rapid mode of action. A similar staining trend is then observed for chloroquine and mefloquine, with mefloquine showing a slightly greater signal at the 8 hour mark. Finally, as

anticipated, doxycycline showed very little antimalarial activity in the final time point as it had done across the PI study.

In a similar study the calcein-AM staining patterns were assessed and showed a similar trend to that of PI, albeit in reciprocal fashion. A general trend of a decrease in calcein staining can be observed with the time progression (Figure 25) and as a direct result of the rate of action of each drug in question.

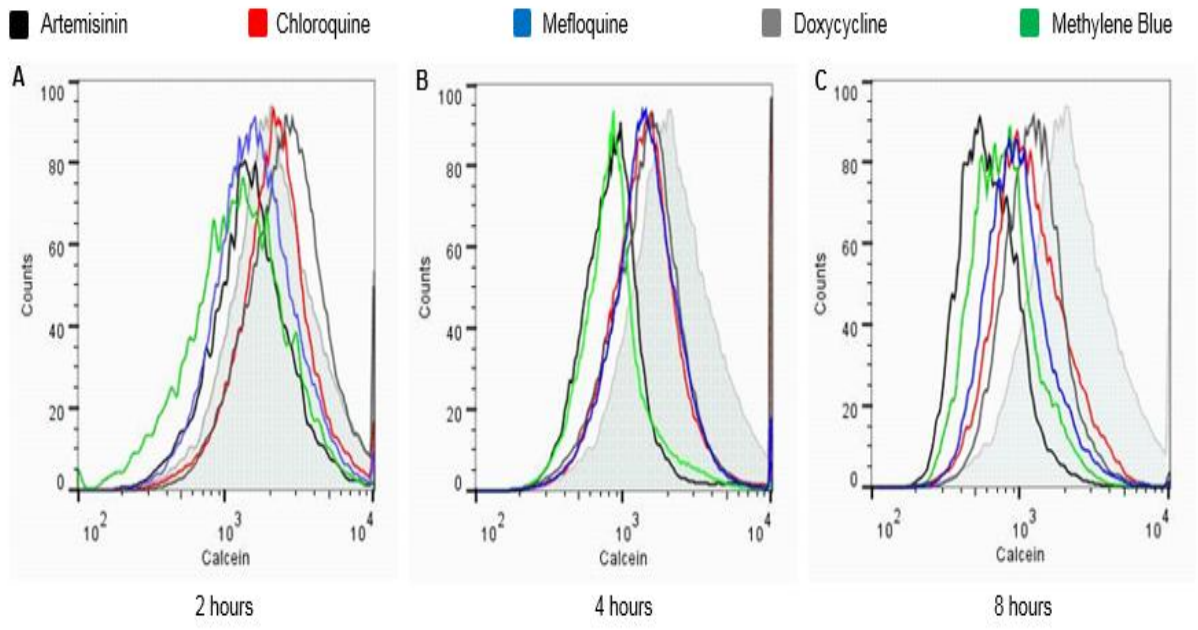

Figure 25: Flow cytometry histogram comparison of the model antimalarial drugs and their respective rates of action as a measure of calcein-AM positive events after being treated for 2 hours (A), 4 hours (B) and 8 hours (C) with Doxycycline (100 µM), chloroquine (120 nM), mefloquine (90 nM) artemisinin (150 nM) and methylene blue (110 nM) respectively. The shaded peak is representative of unstained parasites. Post treatment the samples were stained with PI (3.3 µg/mL) and analysed using FlowJo software (Tree Star Inc.).

While PI staining increases as the parasite population shows signs of increasing stress and eventual collapse, the inverse is true for calcein-AM. Untreated cells are expected to have the highest calcein staining and hence an indication of a healthy population. The 2 hour mark depicts very little change in staining patterns from each drug, as well as in relation to the

untreated sample. This is the same trend experienced from the PI samples and hence suggests that 2 hours is too soon to see any lethal impact on the parasites from the drug compounds. As with PI, the 4 hour mark shows the first signs of a significant shift in fluorescence trends between drugs as methylene blue and artemisinin treated parasites both show decreases in calcein positive staining and almost matching peaks. Chloroquine, mefloquine and doxycycline all show minor decreases in fluorescence at this point as well as comparable peaks. Again, similar to the PI study, the largest variation between the drugs is observed at the 8 hour mark as artemisinin repeatedly shows the quickest rate of action with the most significant decrease in calcein staining. This is apparent across all time points for the artemisinin treated, calcein-AM stained sample. Methylene blue ranks in a close second followed by chloroquine and mefloquine with very similar trends. Finally doxycycline shows very little deviation from the untreated sample showing a predominantly healthy cell culture at 8 hours of drug treatment.

An alternative to visualizing the data is by analysing the fluorescence intensity of the individual probes across the entirety of each drug treated sample. In this way a more convincing conclusion could be made on the sensitivity of both PI and calcein-AM to the rates of the drugs in question. Mean fluorescence intensity across each drug treated sample was then calculated at each time point.

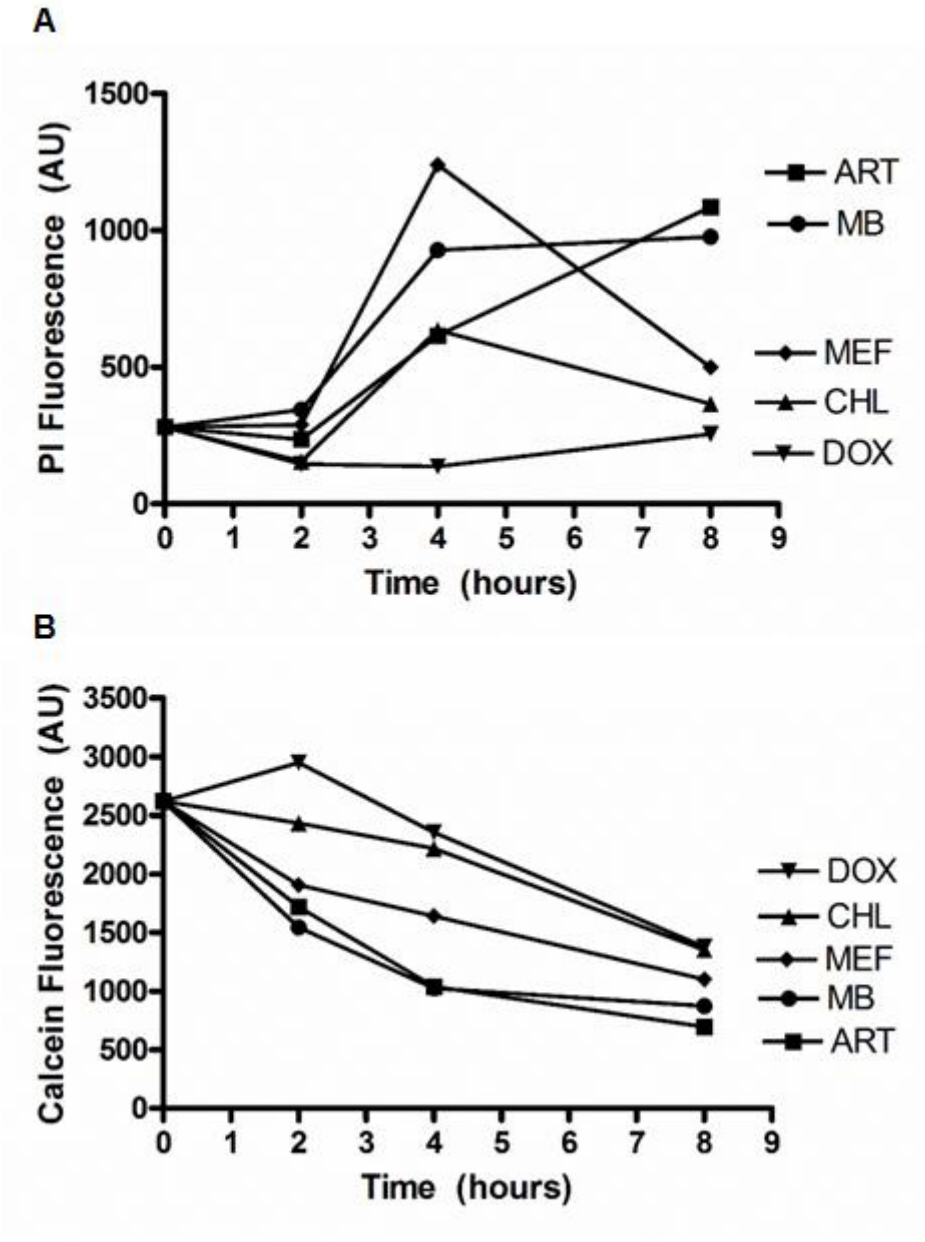

Figure 26: Mean fluorescence intensity of each drug treated parasite sample at the respective time points after FACS analysis. Mean fluorescence intensiy for PI (panel A) and calcein-AM (panel B) channels were obtained from the FACS data using FlowJo software across and plotted against the time intervals for each drug. ART– Artemisinin; MB – Methylene blue; MEF – Mefloquine; CHL – Chloroquine; DOX – Doxycycline. Note: Each data point is representative of one sample per reading.

It can be said that PI (Figure 26 A) provided a more sufficient means of distinguishing between the rate of action of the panel of antimalarials in comparison to that of calcein-AM over 8 hours. Although the contrast was observable to a lesser degree over the calcein-AM study (Figure 26 B) it still provided the same ranking, albeit in reverse.

3.3.2 - Fluorescent Probe Filter Tube Assay

The fluorescence produced by the vitality probes in drug-treated parasites has been shown to be quantifiable by flow cytometry. This provided a precise analysis of how parasite populations respond to drug exposure. However, it also requires access to a flow cytometer therefore the possibility of using a multiwell fluorimeter to quantitate calcein and PI fluorescence in drug-treated parasite populations was explored. This approach is complicated by the fact that RBC's in the culture also accumulate calcein and need to be removed by selective cell lysis (using saponin). This led to the development of a novel method for rapidly removing probe-stained parasites from saponin lysed red blood cells using microfuge tubes fitted with removable 0.45 µm filters (filter tubes) and obtained promising fluorimeter results.

Briefly, a final parasite suspension of 5 % haematocrit and 5 % parasitaemia was used for test samples incubated in their respective drug compounds (10 x IC_{50}) for 8 hours. Gramicidin D (100 pM) was used to simulate a collapsed parasite population and in this case was applied for 1 hour. Post treatment the samples were washed in PBS and stained with calcein-AM (2 µM) for 30 min (all but 'unstained' control) before again being washed twice in PBS. The host RBC's were lysed using a 0.3 % saponin, 0.5 % BSA solution after which the lysates were transferred to a Spin-X HPLC micro centrifuge filter tube. The lysates were then spun down, which caused the calcein which was trapped in the RBC cytoplasm and released by saponin lysis to pass through the pores of the filters, while the calcein-stained intact parasites remained trapped on top of the filters. After discarding the filtrate a solution of 0.5 % Triton-X100 in PBS was used to lyse the parasite cells on top of the filters and release the parasite-associated calcein. The final lysates were transferred to the wells of a 96- well microtiter plate and analysed in a multiwell plate reader to measure residual calcein in each sample.

Fluorescence was measured as Relative Flourescence Units (RFU) and graphed in relation to each drug in the panel (Figure 27).

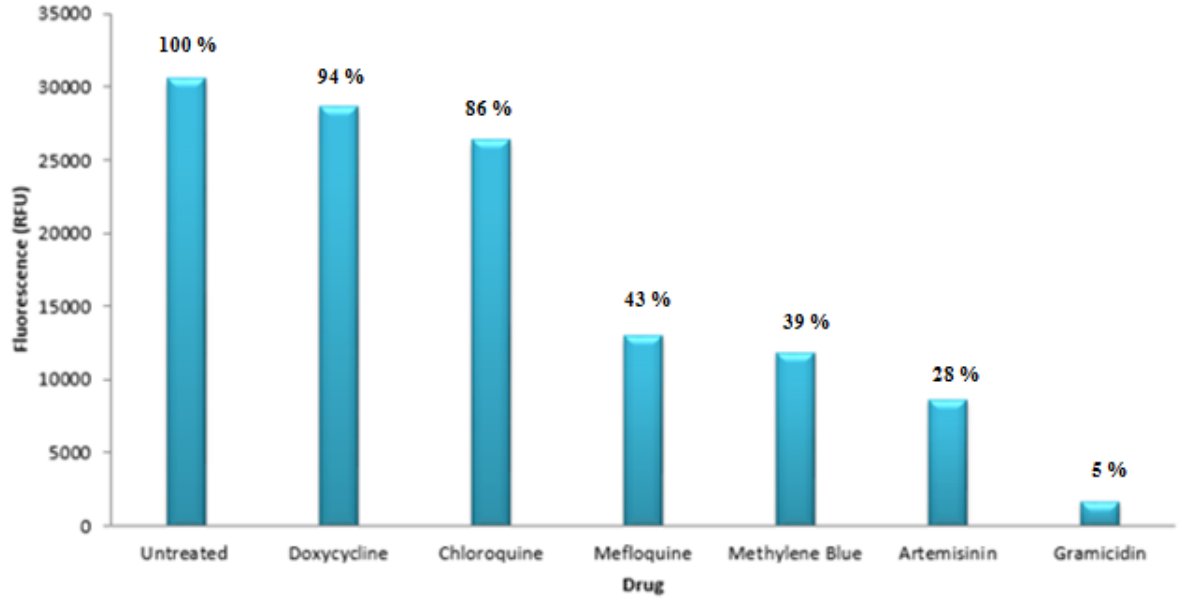

Figure 27: Comparison of the residual fluorescence (as an indication of killing rate) of the panel of antimalarials measured after 8 hours, as calcein-AM relative fluorescence units. Drug test samples were treated for 8 hours and also included an untreated control as well as a 100 pM gramicidin D control to simulate a collapsed parasite population. Percent parasite viabilities are indicated above each drug used which include doxycycline (100 µM), chloroquine (120 nM), mefloquine (90 nM), methylene blue (115 nM) and artemisinin (150 nM). Viabilities were calculated in relation to the untreated control sample which represented 100 % parasite viability. Note: Each viability as a measure of RFU is representative of one sample per reading.

Using the untreated control as a baseline for a healthy, viable parasite culture and the gramicidin D treated sample as an indication of a fully collapsed population of parasites the panel of drugs was ranked according to the degree of residual calcein fluorescence remaining after an 8 hour treatment. Doxycycline showed a minor change in RFU of 6.4 % parasite viability in relation to the untreated sample, with chloroquine showing a 7.4 % reduction in viability in relation to doxycycline. This would suggest these two drugs have very little impact on the parasite samples over an 8 hour time period. Mefloquine and methylene blue show more significant reductions in RFU in relation to the untreated control with viabilities

of 43 % and 39 % respectively. This correlated to a significantly lower residual calcein fluorescence compared to doxycycline and chloroquine. Artemisinin had the lowest RFU out of the test drug samples with 28 % remaining parasite viability after 8 hours of drug treatment and would suggest the fastest rate of action of the panel of drugs. The control treated with 100 pM of gramicidin D proved most effective with only 5 % of parasites remaining after an 8 hour treatment.

3.3.3 - Optimized ATP Assay

In 2012 the Khan group identified the need for a novel, quantitative assay for determining the kinetics of drug action in culture. This came after the announcement from the Medicines for Malaria Venture (MMV) that a radical cure for chronic malaria which could be administered in a single dose (at most a three day therapy) and that could compete with the efficacy of Artemisinin had become a global necessity (Internet 1). Although the PRR assay discussed in Chapter 1 has been a major breakthrough in measuring rate of drug action *in vitro* it remains tedious and low in throughput. Therefore an alternative means of assessing antimalarial drug kinetics in culture was developed using changes in ATP levels as a direct indication of drug pressure. As such they made use of the commercially available CellTiter-Glo Luminescent Cell Viability Assay kit (Promega) to assess the rate of action of a panel of antimalarials by quantifying parasite ATP in drug treated parasite cultures (Khan *et al.,* 2012). Although their study confirmed ATP levels to represent a sensitive and readily quantifiable means of screening the rate of drug pressure on parasites in culture, it was not expected that ATP levels would increase post drug treatment as was evident in the study. The conclusion that the harmful effects of the drugs triggered an increase in metabolic activity and subsequent elevated ATP levels to combat the degradation is questionable and hence was explored in an optimized version of the assay in this chapter. Optimization entailed the use of the microfuge

filter tubes (as described for the fluorescence filter tube assay in the previous section) to remove RBC ATP and allow specific quantitation of parasite ATP.

Similarly to the fluorescence filter tube assay, a final parasite suspension of 5 % haematocrit and 5 % parasitaemia was used for test samples incubated in their respective drug compounds (10 x IC_{50}) for 8 hours. Gramicidin D (100 pM) was used to simulate a collapsed parasite population and was treated for 1 hour. Post treatment the samples were washed in PBS and the host RBC's were lysed using a 0.3 % saponin, 0.5 % BSA solution after which the lysates were transferred to a Spin-X HPLC micro centrifuge filter tube. The lysates were then spun down and after discarding the filtrate (containing the ATP in lysed RBCs), the filters (containing intact parasites) were placed in fresh microfuge tubes and an equal volume of PBS and CellTiter-Glo reagent was added to each filter. The CellTiter-Glo reagent lyses the parasites and the released ATP produces luminescence due to the presence of firefly luciferase and its substrate in the reagent. The lysates were then transferred to the wells of a white-walled, clear bottom 96-well plate and the luminescence of each well was measured in a multi-well plate reader to measure parasite-associated ATP. Luminescence was measured as Relative Luminescence Units (RLU) and is graphically illustrated in relation to each drug in the panel (Figure 28).

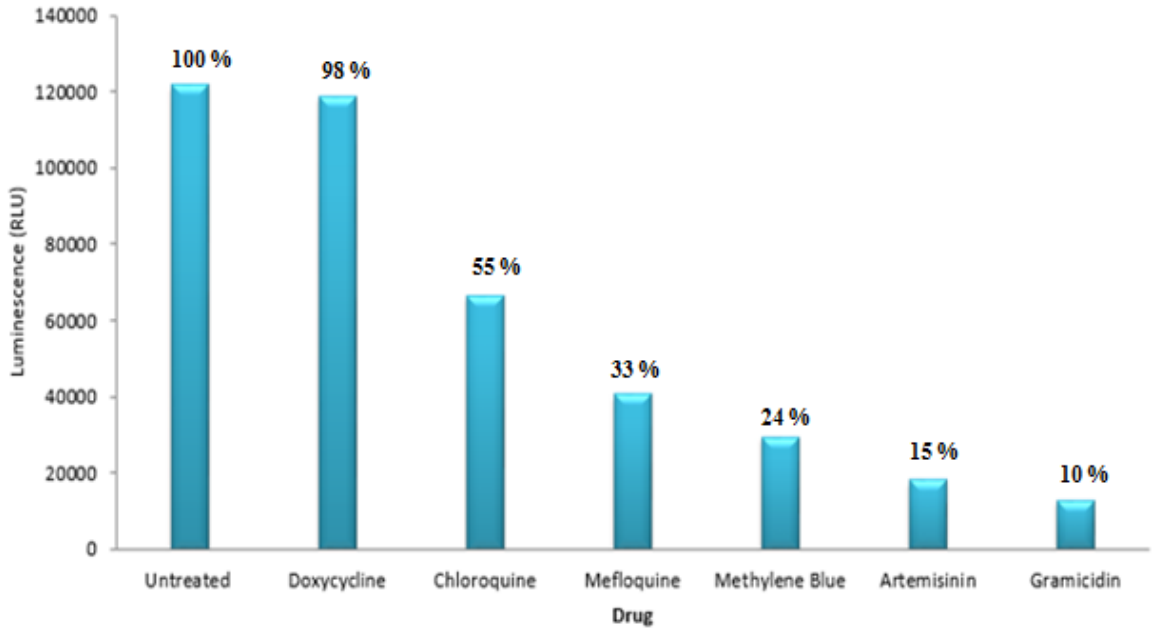

Figure 28: Comparison of the relative luminescence of the panel of antimalarials as measured by relative luminescence units (RLU) which reflects the ATP levels in treated parasites. Drug test samples were treated for 8 hours and also included an untreated control as well as a 100 pM gramicidin D control to simulate a collapsed parasite population. Percent parasite viabilities are indicated above each drug concentration which include doxycycline (100 µM), chloroquine (120 nM), mefloquine (90 nM), methylene blue (115 nM) and artemisinin (150 nM). Viabilities were calculated in relation to the untreated control sample which represented 100 % parasite viability. Note: Each viability as a measure of RLU is representative of one sample per reading.

The untreated control sample acted as a model healthy cell culture and the RLU associated with it was deemed to represent 100 % parasite viability. In contrast the gramidicin D treated sample was indicative of a parasite population under extreme stress and its related RLU was evident of this with only 10 % remaining parasite viability (defined in this case as the level of residual ATP in the parasites compared) in comparison to the untreated control. Doxycycline appeared to have little effect on the parasites with an almost entirely viable cell culture of 98 % after 8 hours of drug treatment. Chloroquine and mefloquine appeared to have significantly more impact with 55 % and 33 % parasite viability respectively. Methylene blue and artemisinin provided the quickest rates of action with only 24 % and 15 % remaining parasite viability respectively after 8 hours of treatment.

3.3.4 - Parasite Reduction Ratio Assay

To be able to confidently determine whether the reductions in parasite viability observed in the fluorescence microscopy, flow cytometry, fluorescence filter tube and ATP assays were indeed indicative of compromised parasite viability, a modified PRR assay was conducted. The principle of the PRR assay is based on the fact that the asexual parasite life cycle lasts for 48 hours and as such the assay ultimately determines what percentage of a population of drug treated parasites are able to recover from a limited period of drug exposure (8 hours in this study) in a single life cycle. This represents a modification of the originally described PRR assay (Sanz *et al.*, 2012) in the sense that the original method uses longer exposure times as well as recovery times before quantifying the recovered prasites.

Early trophozoite stage cultures were treated with each drug as per the previous assays at 10 x the IC_{50} of each respective drug for 8 hours. Following treatment, the drugs were washed from each sample and fresh CM was added to each drug-free culture and returned to incubation in the special gas mixture at $37^{o}C$ for the full 48 hour period. After the final incubation period the cultures were assessed for parasite viability using the pLDH assay shown in Figure 29.

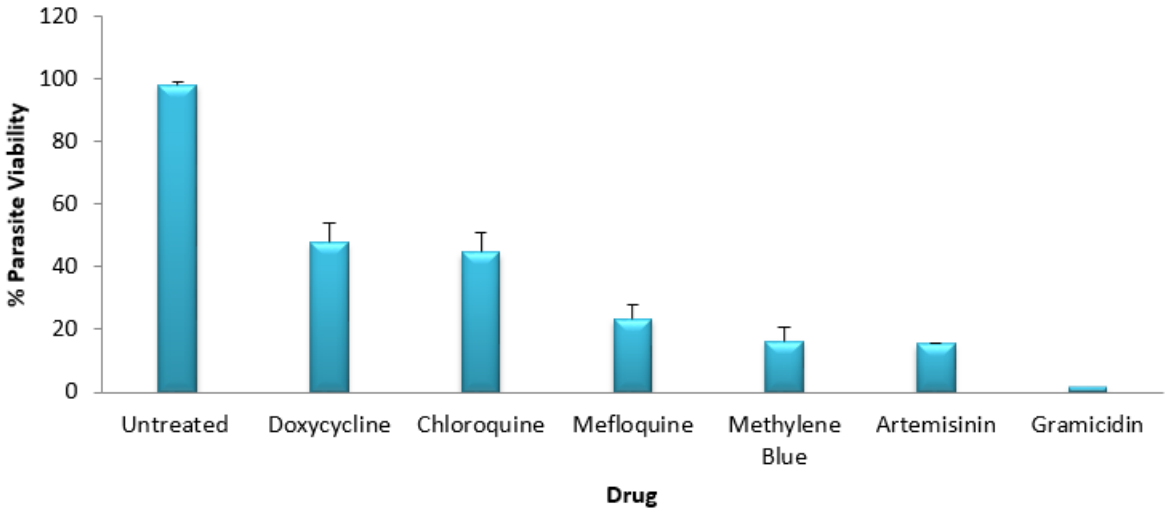

Figure 29: Percentage remaining parasite viability for each drug in the panel measured using the pLDH assay in early trophozoite-stage parasite cultures after 8 hours of drug treatment and 48 hours after removal of the drug. Each data point represents mean +- standard deviation. P-values were calculated for each remaining drug-treated viability in relation to the untreated control and in all instances $p < 0.01$ ($N = 5$).

After the 48 hour incubation period the gramicidin D treated control sample showed a negligible parasite viability of 2 % in relation to the uUltimately, the differences observed in percentage parasite viabilities of the panel of drug treated samples after 8 hours of treatment allowed for a comprehensive ranking system (Table 5) to be considered. Interestingly, although the variations in viabilities for each drug were extensive across the assays performed, the ranking of each drugs rate of action in the respective assays (fluorescence microscopy, flow cytometry, filter tube fluorescence, ATP and modified PRR assays) remained essentially the same: artemisinin > methylene blue > mefloquine > chloroquine ~ doxycycline.

3.4 - Discussion

3.4.1 - Quantifying the Fluorescence Assay using Flow Cytometry.

The fluorescence microscopy based bioassay developed in Chapter 2 was able to assess the changes in parasite staining patterns after 8 hours of drug treatment by counting the ratio of viable (calcein positive/PI negative) vs. non-viable (calcein negative/PI positive) parasites in each sample. This method, although quicker to perform than the modified PRR assay, provided only semi-quantitative rankings of the rate of each drug in the panel. To improve quanitification the novel assay was adapted for analysis via flow cytometry.

Flow cytometry has long since been established for use as a reliable and rapid method for parasite detection in human blood or malaria culture samples (Pattanapanyasat *et al.,* 1992) as well as in *Plasmodium*-infected mice (Barkan *et al.,* 2000). More recently this technique has been used to measure individual drug efficacy against the *P. falciparum* parasite. In the parasite sexual life stage, gametocytes of transgenic GFP-expressing parasites of the 3D7 laboratory strain were detected and a panel of drugs, including chloroquine, were used to assess drug pressure via flow cytometry (Wang *et al.,* 2014). Even more relevant is the fact that fluorescent probes have been used as indicators of drug pressure for specific anti-gametocyte drugs using hydroethidine whereby only viable parasites retained the probe and inhibitory drug concentrations were assessed (Chevalley *et al.,* 2010). Previous studies have been conducted making use of various fluorescent probes to measure parasitaemia of the *P. falciparum* asexual life stage using flow cytometry and these include Hoechst 33258 (Brown *et al.,* 1980), acridine orange (Whaun *et al.,* 1983), thiazole orange (Makler *et al.,* 1983) and hydroethidine (Wyatt *et al.,* 1991). However these assays have simply been as a screen for the detection of the parasite asexual blood stage and as recently as 2014 are still being compared and contrasted to the Giemsa stained microscopic examination of blood smears

(Jang *et al.*, 2014). This study made use of flow cytometry to develop a more rapid and quantitative method for assessing the rate of action of antimalarial drugs using the vitality probes PI and calcein-AM.

After providing promising analysis via fluorescence microscopy the calcein-AM and PI probes were adapted for use in flow cytometry and used to assess the same panel of drugs used in the previous assay. Used in combination with each other as well as with the now included Hoechst 33342 dye provided confirmation of the results observed for the fluorescence microscopy assay i.e. the fluorescent vitality probes PI and calcein-AM are sensitive enough to distinguish between live and dead parasites when used in conjunction to measure antimalarial killing rates. With the fact that the drug concentrations of the panel as well as the probe staining concentrations remained identical to that of the fluorescence microscopy assay, direct comparisons could be made. The ranking of the panel of drugs mirrored that of the microscopy assay (besides the addition of methylene blue in the flow cytometry assay) i.e. artemsinin > methylene blue > melfoquine > chloroquine > doxycycline.

A minor note would be that in assessment of the calcein-AM positive events it appeared that and chloroquine had almost identical rates of action at the 4 and 8 hour marks, a trend that was not observed with the PI probe. It is therefore important to understand the necessity of using both PI and calcein-AM probes when distinguishing between antimalarial killing rates of similar drugs. If the results depicted from calcein-AM visualization are taken at face value they could allow for the assumption that mefloquine and chloroquine have similar if not the same rates of action. Furthermore if inferring mechanism of action of the drugs is pursued, the fact that the rates are the same may then argue for similar mechanisms such as the enhanced binding of haem to the parasite membrane (Loria *et al.*, 1999).

In comparing the two probes, PI and calcein-AM, and assessing which provided the more accurate distinction between the panel of drugs as a measure of parasite killing rate it could be argued that PI was the more sensitive vitality probe. The exponential difference in rate of drug action after 8 hours of treatment provided a more confident ranking to be achieved in comparison to that of calcein-AM. This would be due to the fact that during fluorescence microscopy analysis it was observed that PI was only capable of staining parasite cells that had significantly compromised cellular vitality and hence provided a more adept means of counting nonviable parasites. Calcein-AM, although only able to probe for viable parasites, may still provide residual fluorescence that is detected by the flow cytometer as a positive event in dying cells. This would make the distinction between the rates of the drugs over shorter incubation periods less pronounced, as was observed.

Another observation was the apparent anomaly at the 4 hour drug treated time point for the PI probe. At the 2 hour treatment point all the drugs showed a relatively similar number of PI positive fluorescent events. However at the 4 hour mark a spike in fluorescent events was witnessed in the mefloquine and chloroquine treated samples that was not apparent in the other drug samples. Although this could be perceived to be an apparent outlier it is curious that the spike was observed in both quinine derived drugs. Alternately, this could be an indication of cellular lysis or apoptosis after this time point and hence far fewer PI positive events at the 8 hour mark. The fact that the rates of action appeared to normalise at the 8 hour mark, a perceivably more accurate time point to make assessments of the ranks of rate of drug action, this irregularity was not further pursued

3.4.2 - Adaptation for Use in a Mutiwell Fluorimeter

The fluorescence produced by the vitality probes in drug-treated parasites was efficiently quantitated using flow cytometry which provided a detailed and accurate analysis of how individual parasites cope with drug pressure. The single downfall to the current assay is the requirement of a flow cytometer. Therefore to produce a more "user-friendly" assay for wider dissemination the possibility of using a multiwell fluorimeter to quantitate calcein fluorescence in drug treated parasite populations was explored. This approach was complicated further by the fact that uninfected RBC's also accumulate calcein and need to still be removed by selective cell lysis using saponin. Therefore a novel method for rapidly removing probe-stained parasites from lysed RBC's using filter tubes was considered.

Two issues arose with the addition of the filter tubes to the assay. First, anymore than a total of 5 µL total volume added and the filter would become clogged. Whether this was a clogging due to cellular debris post saponin lysis or simply due to overload of the membrane is unclear. Therefore it was crucial to ensure any staining, saponin lysis and subsequent washes were conducted in a volume of 5 µL or less per application. This potential hindrance was not unforeseen, however, as the centrifuge filter tubes employed for use in the assay are most commonly used in pharmaceutical studies in the isolation of smaller particles such as drugs and other inorganic matter and not necessarily for organic substances such as residual calcein fluorescence isolated from whole parasite cells (Velmurugan and Selvamuthukumar, 2014).

Second, the RFU read outs were erratic across replicates with little reproducibility across separate experiments. The viabilities depicted in this study for the filter tube assay are representative of the single most efficient experiment. The study therefore culminated in achieving parasite viabilities post drug treatment that differed slightly from that observed

from the fluorescence microscopy and flow cytometry assays but importantly provided the same rate of action ranking i.e. artemsinin > methylene blue > melfoquine > chloroquine > doxycycline (although this ranking was a representation of one experiment). These rates were in relation to an untreated control with the gramicidin D treated sample representing a control with the lowest parasite viability after 8 hours of drug treatment. Nonetheless, the assay will require further development to circumvent the filter clogging problem. One possibility is to use filters with larger pore sizes (e.g. 1-2 µm as opposed to the 0.45 µm used here). There are other means for rapidly removing the RBC lysate from saponin released parasites, notably centrifugation through a phthalate oil mixture (e.g. Khan *et al.*, 2012). However, the filter tube method was pursued since it could in principle be adapted to an improved throughput assay, by using 96-well plates with filter plate inserts.

3.4.3 - Optimization of the ATP assay

In eukaryotic cell systems ATP production is required for use as the principal energy source for growth and maintenance. Concisely, glucose uptake and ensuing conversion to pyruvate during glycolysis initiates the mechanism. Pyruvate is subsequently converted to acetyl-CoA in the mitochondria which is then oxidised to CO_2 via oxidative phosphorylation resulting in the production of ATP, among other biosynthetic precursors. This process is significantly less efficient in the intra-erythrocytic life stage of the *Plasmodium* parasite whereby it lacks the mitochondrial complexes required for aerobic production of ATP. Instead the parasite metabolises glucose anaerobically and pyruvate is fermented to lactate to produce the required ATP for energy (Olszewski and Llinas, 2011).

The need for a novel, quantitative assay which is capable of determining the kinetics of drug action in culture as well as being more efficient in comparing the efficacy of novel antimalarials to existing drugs was identified by the Khan group in 2012. As such a focus was

placed on identifying whether parasite ATP levels may represent a sensitive and readily quantifiable means of diagnosing associated drug pressure. After experimental refinement a rapid, functional ATP assay was developed which provided evidence during time-course treatments that it was able to assess the rate of activity of antimalarial drugs by measuring ATP levels in drug-treated cultures (Khan *et al.,* 2012). However, the elevated ATP levels observed post drug treatment that were attributed to an increase in metabolic activity requires further review. Furthermore the assay protocol also had areas for refinement as well as adaptation e.g. via the use of the filter tubes employed in the adapted fluorescence assay produced in this study.

In the initial pilot assay conducted by the Khan group (2012) a total of five wash steps were required to isolate the intact parasites from the RBC's post saponin treatment. This process although necessary was tedious and time-consuming and hence a phthalate oil-mix was used as an alternative method for parasite isolation from RBC lysates (Khan *et al.,* 2012). This procedure reduced the number of wash steps to two and additionally increased the amount of residual RBC ATP retained in the samples. However the addition of the phthalate oil-mix to isolate parasites from RBC's was not completely effective and increases in saponin concentration (0.1 % to 0.24 %) as well remaining RBC 'ghosts' were still an issue in the final protocol. This study therefore proposed eliminating the phthalate oil-mix isolation of parasites from their respective RBC hosts and instead making use of 0.45 µm nylon filters for isolation of the parasites post drug treatment and prior to the addition of the CellTiter-Glo reagent. As with the fluorescence assay discussed above, the use of the filter tubes was further motivated by an exploration of the feasibility of using 96-well filter plates to improve throughput of the ATP assay.

The filters themselves brought the same issues as was discussed with the fluorescent filter tube assay. Any more than 5 µL in a single application and the membrane would become clogged and not allow the filtrate to pass through. Furthermore, repeat assays provided erratic results and results provided in this study are indicative of the most successful assay. Ultimately a ranking of the drugs in relation to their individual killing rates as a measure of residual ATP was achieved albeit as a representation of one sample per reading. The most successful assay was agreeable with the flow cytometry, fluorescence microscopy and fluorescent filter tube assays i.e. artemsinin > methylene blue > melfoquine > chloroquine > doxycycline.

There is sufficient published evidence that artemisinin is a more rapid acting drug in relation to other antimalarials and has been compared and proven to be faster acting that both chloroquine and mefloquine both *in vitro* (Sanz *et al.*, 2012; Khan *et al.*, 2012) and *in vivo* (Myint and Shwe, 1987; Karbwang *et al.*, 1992; White, 1994). Although mefloquine has been proposed to share many of its antimalarial properties with chloroquine its precise mechanism of action is still unclear, whereas chloroquine activity is now generally accepted to inhibit haem detoxification. It is therefore unclear as to why elevated ATP levels were observed post drug treatment in the Khan study and their assumption was that it reflected increased metabolic activity by the parasite as part of its stress response in order to overcome detrimental drug effects. This can be supported by the theory that a decrease in kinase action because of direct/indirect inhibition of kinases, or total loss of systems using ATP. The increase of metabolism producing ATP would be to cope with any stress or cellular damage (Barouch-Bentov, 2011).

An 8–hour treatment of artemisinin resulted in significantly lower ATP levels in relation to chloroquine and mefloquine, a trend that was opposite in the Khan study. This study would

therefore then suggest that antimalarial drug pressure results in reduced parasite metabolic activity and hence reduced ability to produce and maintain ATP levels. It is unclear whether *Plasmodium* parasite associated ATP levels have been measured as an indication of drug stress in other studies other than the Khan group. However, ATP levels have been used as indication of selective drug pressure in *Leishmania* parasites and similar results to this study were observed – ATP levels, as well as overall mitochondrial membrane potential, were reduced in drug treated parasites in relation to controlled wild-type models (Garcia-Hernandez *et al.*, 2012). This would then correlate to the decrease in ATP levels observed in this study as a result of drug pressure over an 8-hour time period.

3.4.4 – Parasite Reduction Ratio Assay

Although other approaches to measure malarial killing rates have been documented (Young and Rathod, 1993; Painter *et al.*, 2010) they are limited in the key factor of determining viable parasites over the observable period of growth during drug treatment and analysis. As such the number of parasites that survive drug treatment and are able to resume productive growth and replication remained unaccounted for after the removal of the drug and addition of new RBC's to culture. The PRR assay was a major breakthrough in measuring a definitive killing rate of novel antimalarials in relation to standard drugs by measuring drug pressure over broad time courses and then accounting for any regrowth observed after an additional asexual life cycle (Sanz *et al.*, 2012). The advantage of this method is i) it does not rely on the drugs mode-of-action for the measurement of killing rate and ii) allows a direct comparison of antimalarial killing rates based on individual drug IC_{50} values. Although essentially definitive, this protocol remains time consuming and labour-intensive in its approach, as well as being significantly low in throughput. Although the assays presented in this study aim to improve on these short comings it was necessary to compare *in vitro*

antimalarial killing rates observed via the novel assays to the PRR assay to gauge their efficiency in relation to the current gold standard in measuring drug killing rates on Plasmodium culture. As such the assay was modified slightly and the ranking of the panel of standard antimalarials was directly compared to the novel fluorescence microscopy, flow cytometry, fluorescence filter tube and optimized ATP assays.

The parasite viabilities observable a full life cycle after removal of the drug and measured via the pLDH assay provided the two most convincing control samples of all the assays. Artemisinin showed the closest killing rate to that of gramicidin D albeit fractionally faster than that of methylene blue. The sample treated with mefloquine was only able to recover a quarter of the viable parasites in the population which correlated almost identically to what was observed in the ATP assay. Similarly only half the population was able to recover after chloroquine treatment, which again corroborated the results observed in the ATP assay providing evidence of the efficiency of this assay, if at the very least for the determination of the killing rates of the quinine derived drugs in the panel.

An interesting observation was the recovery rate of doxycycline. The parasites able to recover from doxycycline treatment was only 1 % more than that observed post chloroquine treatment. In contrast the viabilities observed for doxycycline in the novel assays produced in this study showed very little visible effect on the parasite population after 8 hours of drug treatment. In the case of the ATP and fluorescence filter tube assays there was little to no significant difference from that of the untreated control. This may present an issue in determining the killing rates of slower acting antimalarial drugs such as doxycycline that only show visible effect on parasite populations in the life cycle immediately following that in which drug pressure is applied. This is not an impediment in terms of the purposes of this

study in which the aim was to achieve a ranking of antimalarials such that the most rapid can be identified.

Ultimately the modified PRR assay was efficient in measuring the net effect of drug pressure on parasite viability and allowed for the ranking of a panel of standard antimalarials used in the development of the novel assays in this study. More importantly, although the individual parasite viabilities differed in some cases to the novel assays, the ranking achieved post assay remained the same i.e. artemsinin > methylene blue > melfoquine > chloroquine > doxycycline.

3.5 - Conclusion

In order to increase throughput and quantification of the fluorescence microscopy bioassay it was adapted for use in flow cytometry. This adaptation led to pilot assays being conducted and identified the need for the addition of the Hoechst 33342 nucleic acid probe in order to gate the fluorescence readings correctly and isolate the signals obtained from parasites from residual unparasitized RBC's post saponin lysis. This allowed for any analysis and inference to be made solely based on parasite associated staining patterns. The pilot assays showed evidence that PI was capable of adequately and repeatedly distinguishing between viable and non-viable parasites, however, calcein required minor optimization in the form of an additional saponin lysis step to remove the intact parasite from their host RBCs (due to the inherent nature of RBCs to retain fluorescent calcein). The subsequent time course assays gave preliminary insight into the killing rates of a panel of standard antimalarials, although these rates were less clear at the 2- and 4-hour time points than at 8-hours post treatment. As 8-hours of drug treatment was the last time point in this study it would be useful to extend the time course in order to better separate the drugs based on parasite killing rates using flow cytometry and therefore gain a better understanding of the individual rates of action. It was

found that the ranking of the panel of drugs mirrored that of the microscopy assay i.e. artemsinin > methylene blue > melfoquine > chloroquine > doxycycline.

The addition of nylon filter tubes in order to explore the possibility of adapting the fluorescence microscopy and flow cytometry assays for use in a multiwell fluorimeter was met with significant complications. The issues of membrane clogging and erratic results across replicate assays was difficult to overcome and as a result only a single effective assay was used to provide a ranking of the antimalarials used in this study for the filter tube assay. Furthermore the clogging of the membrane (any more than a 5 µL application) could potentially prevent residual fluorescence from drug treated parasite samples from passing through the filter and entering the filtrate that is analysed. This would account for the erratic RFU observed over repeat experiments. Larger pore size filters can be explored since it may allow other organic matter such as cellular debris from saponin lysis to escape more efficiently into the filtrate prior to analysis. Therefore, although the results presented herein depict an agreeable assay to the fluorescence microscopy and flow cytometry assays in terms of the ranking of the panel of drugs, it cannot be said with confidence that this procedure has been optimized sufficiently to provide a robust method of measuring the killing rate of standard or novel antimalarials on a malaria parasite population.

The results from the optimized ATP assay using filter tubes to separate intact parasites from RBC contaminants suggested the same trend observed with the fluorescence filter tube assay. Although the most efficient assay was exhibited in this study and agreed with previous assays produced, replicate assays did not provide sufficiently robust outputs to confidently declare the ATP assay (as it currently stands) to be a suitable indicator of the killing rates of a panel of antimalarials. As with the fluorescence filter tube assay, membrane clogging may be a key factor in this issue preventing residual ATP post drug treatment from entering the filtrate past

organic matter and hence providing skewed luminescence readings. Furthermore, ATP levels decreased post drug treatment and did not correlate to the increase in ATP that the Khan group (2012) observed thereby suggesting that parasite ATP levels had been compromised directly by antimalarial drug pressure. Due to the lack of reproducibility it cannot confidently be said that this study provides evidence to contradict the validity of the assay produced by the Khan group.

It can be argued that the modified PRR assay provided the most accurate ranking of the antimalarials as the assay was reproducible and robust in this study and provided a definitive ranking of the panel of drugs. Importantly, it accounted for the efficacy (albeit slower than rest of the drugs in the panel) of doxycycline after the 48-hour recovery period from drug treatment – a feature that was noticeably absent from the novel assays produced in this study. Ultimately, although the antimalarial killing rates differed in some cases to the novel assays, the ranking achieved from the PRR assay remained the same as the novel assays i.e. artemsinin > methylene blue > melfoquine > chloroquine > doxycycline.

Chapter 4 – General Conclusion and Future Prospects

Victims of acute malaria, especially in third world countries, rely profoundly on drugs with a rapid onset of action, where the use of compounds with longer acting half-lives can lead to the onset of the severe symptoms and even death. The additional complication of patient compliance through the full course of drug treatment only further enhances the need for a single dose, rapid acting antimalarial drug. This points to a need to accurately and quantitatively determine the rate of drug action on malarial parasites with a priority to match or exceed the rate of action of artemisinin and its current combination therapies, the current gold standard in malaria treatment.

The MMV have endorsed a single-dose radical cure as the ideal treatment for uncomplicated malaria whereby reductions in cost and relief from patient noncompliance would greatly reduce the risk of drug resistance. Until recently the rate of drug action has been measured by light microscopic examination of parasite morphology using blood slides of drug treated parasite cultures at regular time intervals. This technique is tedious and, most importantly, subject to interpretation with regards to distinguishing between viable and comprised parasite cells, thus making it impossible to objectively quantitate the rate of drug action. What is needed is a means to unambiguously quantify viable and non-viable/compromised parasites in cultures exposed to drugs. The modified versions of the PRR viability based assay which determines this rate by directly quantifying the number of parasites capable of recovering from distinct periods of drug exposure (Sanz *et al.,* 2012) is time-consuming and low in throughput. Although recent developments have provided more adept means of measuring *in vitro* killing rates of antimalarials via the ATP assay (Khan *et al.,* 2012) there remained questionable outcomes and areas for assay improvement. This study developed a series of novel bioassays using the calcein-AM and PI fluorescent vitality probes which allowed the

rate of drug action on *P. falciparum* malaria parasites to be assessed and ranked in relation to each other. Importantly these assays made the assessment of the killing rates i) more rapid; ii) quantitative and iii) adaptable to further increased throughput.

A rapid protocol for measuring *P. falciparum* viability in culture using the fluorescent vitality probes PI and calcein-AM was developed and optimized. It was shown that not only were the probes capable of providing definitive conclusions on the vitality of healthy and drug-treated mammalian HeLa cells (as they are marketed to do) but that these staining patterns were reproducible in malaria parasites. Using fluorescence microscopy and ImageJ for analysis a panel of standard antimalarial compounds was semi-quantifiably ranked according to parasite killing rate. Importantly this ranking concurred with the rates expected from known literature and provided confidence in the novel pilot bioassay. Furthermore, the probes were able to be used in conjunction with each other, showing no cross-over staining, such that a more confident representation of the morphological status of fields of cells could be determined. Ultimately it was observed that over longer time periods and using quicker acting drugs, like artemisinin, a decline in calcein fluorescence and corresponding increase in PI fluorescence marked a deterioration in the overall health of the parasite cell culture. Extrapolation of the pilot assay for use in flow cytometry provided an assay procedure that was capable of measuring the changes in staining patterns in a fully quantifiable manner over 2, 4 and 8 hour time periods. The panel of antimalarial compounds ranked via fluorescence microscopy was found to have the same ranking via flow cytometry – a significant milestone in the production of the final bioassay.

In addition, the flow cytometry results provided significantly different rates of action for the two quinine derived drugs – chloroquine and mefloquine. Hence it could be argued that these two drugs do in fact have differing modes of action – a topic of debate still among

antimalarial drug experts. Therefore this assay has the potential to rank current and novel drugs not only via their individual killing rates but also may be able to group compounds based on similar mechanism and hence infer mode of drug action based on current known drug mechanism.

The viability probe assay as it currently stands (microscopy and flow cytometry) represents an improvement on other assay formats in terms of rapidity and quantification of live/compromised parasites in cultures. However, it requires further development to increase throughput and approach the ultimate goal of producing a medium-throughput assay for rapidly assessing the rate of action of antimalarial drugs. This would require the assay to be adapted to 96-well (or higher format) plates, coupled with the use of a multiwell plate flow cytometer to analyse the probe-stained samples. These improvements may include but are not limited to i) modifying the gating parameters such that parasites are better isolated for analysis, ii) increasing the number of time points over and above 8 hours to gain better insight into the ranking of the drug killing rates over longer treatments.

As these probes provided such conclusive rankings of the killing rates of the standard antimalarials (and that the staining patterns mirrored those observed in HeLa cells) it is an attractive proposition that the novel bioassay could be used in different cancer cells lines to provide a ranking of the killing rates of current and novel cancer drugs for use in chemotherapy. Chemotherapy drugs are initially distinguished via their ability to either act on cancer cells during cellular division or when the cells are at rest and then are subsequently further grouped based on additional properties. Therefore the assay generated in this study could be used not only in malaria parasites but in cancer cell lines to i) quantifiably rank novel drugs based on their killing rates and ii) group novel compounds based on their synonymous mechanism. This theory is currently being pursued using the fluorescent probe

bioassay coupled with flow cytometry in an attempt to identify and rank rapid, novel artemisinin analogs obtained from Prof. Richard Heyns (North West Univeristy) and a group of kinase inhibitors from Dr. Amanda Roussouw (University of Witwatersrand).

Adaptation of the bioassay for use in a multiwell fluorimeter coupled with filter tubes to produce a more 'user-friendly' assay provided precarious results. RFU readings from repeat experiments were erratic and a robust ranking of the antimalarials from the assay was unable to be attained. Similarly, reproduction of the ATP assay generated by Khan and colleagues provided questionable data whereby RLU measurements across experiments were inconsistent. This was definitely a result of the inclusion of the filter tubes in the assays which provided complications from the start. It could be argued that they be removed from the assay procedure, however this would defeat the ultimate goal of producing a medium-throughput bioassay using the same filters in 96 well-plates. Furthermore the increase in ATP post drug treatment that was observed by the Khan group was not observed in this study – perhaps due to optimizations attempted on the original assay i.e. removal of the phthalate oil step and inclusion of the filter tubes.

Finally it can be said that a more rapid, quantitative method for assessing the rate of drug action in *P. falciparum* parasite *in vitro* was generated using vitality probes in combination with fluorescence microscopy and/or flow cytometry. The inclusion of this novel bioassay in the antimalarial drug discovery arsenal will allow for more rapid-acting antimalarials to be identified and more confident assessments made over the traditional morphological assessments that provide flawed, subjective, low- throughput interpretations.

Appendix

1. Thawing Solution A – 10 % NaCl solution

In 1 mL of sterile water, 100 mg of NaCl (Sigma-Aldrich, Germany) was solubilised and filter sterilised in a 0.22 µm filter and stored at 4^{o}C.

2. Thawing Solution B – 1 % NaCl solution

In 5 mL of sterile water, 50 mg of NaCl (Sigma-Aldrich, Germany) was solubilised and filter sterilised in a 0.22 µm filter and stored at 4^{o}C.

3. Fresh RBC's

Blood was extracted from volunteers in SGVac K2EDTA 9 mL collection vials after ethical approval was signed by the volunteer using compliance forms approved by the Rhodes University ethics committee. Immediately after extraction the blood was transferred to a 50 mL falcon tube and placed at 4^{o}C until two layers were clearly visible – the serum layer containing plasma and white blood cells and the lower layer containing the fresh RBC's. The serum layer was aspirated leaving behind the RBC pellet. The pellet was washed in CM and centrifuged at 3000 rpm for 5 min to further isolate and remove any residual serum. A 2 mL layer of CM was left above the fresh RBC pellet and the tube was placed at 4^{o}C for use up to two weeks.

4. Complete Culture Medium – CM

In a sterile 50 mL falcon tube, 2 g of glucose (Sigma-Aldrich, Germany) and 2.5 g of AlbuMax II (Life Technologies, USA) were solubilised in 50 mL of RPMI 1640 medium with 25 mM Hepes and 2 mM L-glutamine (Lonza, Belgium). In an Eppendorf tube, 44 mg of Hypoxanthine (Sigma-Aldrich, Germany) was added to 1 mL of 1 M NaOH before being

transferred to the 50 mL falcon tube. After the glucose and AlbuMax had completely solubilised, 300 µL of Gentamicin (50 mg/mL – Lonza, Belgium) was added and the final solution filter sterilized through a 0.22 µM filter before being added to 450 mL of RPMI 1640 medium and stored at 4^{o}C.

5. Giemsa Stain

A 5 % working solution of Giemsa was prepared from 5 mL Giemsa solution (Sigma-Aldrich, Germany) in 45 mL of 1 X phosphate-buffered saline in a 50 mL falcon tube and wrapped in tin foil to minimise light exposure.

6. Sorbitol solution – 5 %

In a 50 mL falcon tube, 2.5 g of D-Sorbitol (Sigma-Aldrich, Germany) was solubilised in 50 mL of distilled water and filter sterilized through a 0.22 µM filter before being stored at 4^{o}C.

7. Percoll solution – 60 %

A 12.5 % sorbitol/2.5X RPMI1640 solution was prepared by solubilizing 1.045 g of RPMI 1640 powder (Sigma-Aldrich, Germany) and 5 g of D-sorbitol (Sigma-Aldrich, Germany) in 40 mL of sterile water before filter sterilization through a 0.22 µM filter. Thereafter, 6 mL of Percoll (GE Healthcare, Sweden) and 4 mL of the 12.5 % sorbitol/2.5X RPMI1640 solution was prepared and stored at 4^{o}C.

8. Drug Stock Solutions

A panel of standard antimalarial drug compounds were purchased from Sigma-Aldrich, Germany and solubilized to a concentration of 20 mM as per the following profile – chloroquine and doxycycline in sterile water, mefloquine and artemisinin in methanol, methylene blue in ethanol and gramicidin D & S in DMSO. These stock solutions were stored

at -20^{o}C. Intermediate solutions were prepared on the day of use as per the IC_{50} profile of each drug.

9. Malstat solution

In 80 mL of sterile distilled water, 4 g of L-lactate (Sigma-Aldrich, Germany), 1.32 g of Trizma base (Sigma-Aldrich, Germany), and 22 mg of 3-acetylpyridine nicotinamide adenine dinucleotide (APAD; Sigma-Aldrich, Germany) was solubilized. The pH of the solution was adjusted to 9 and 400 µL of Triton-X 100 was added. The volume was adjusted to 200 mL and stored at 4^{o}C.

10. NBT/PES solution

In 100 mL of sterile distilled water, 160 mg of nitro blue tetrazolium (NBT; Sigma-Aldrich, Germany) and 8 mg of phenazine ethosulphate (PES; Sigma-Aldrich, Germany) was solubilized and stored at 4^{o}C in a Schott bottle wrapped in tin foil to minimize light exposure.

11. CellTitre-Glo reagent

The CellTitre-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI) provided a buffer and substrate which were combined and aliquoted equally into Eppendorf tubes. The aliquots were stored at -20^{o}C in tin foil to minimize light exposure.

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