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High Gradient Magnetic Separation (HGMS) of Erythrocytes Infected with Plasmodium Falciparum

Hochgradienten-Magnetseparation (HGMS)
von Plasmodium Falciparum infizierten Erythrozyten

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a	diameter of particle
AB-	blood antigens
AO	Acridine Orange
ARDS	acute respiratory distress syndrome
BSA	bovine serum albumin
$CD4^+$	cluster of differentiation 4^+
CO_2	carbon dioxide
DDT	dichlorodiphenyltrichloroethane
DNA	deoxyribonucleic acid
dr/dt	velocity of fluid
ER	endoplasmic reticulum
ESR	erythrocyte sedimentation rate
Fc region	fragment crystallisable region
F_d	drag force
Fe	iron
F_g	gravitational force
f_{late}	fraction of late-stage infected erythrocytes
F_m	magnetic force
F_p	particle force
f_{ring}	fraction of ring-stage infected erythrocytes
FSC	forward scatter
g	gravitation
G	gauge
h	hours
H	magnetic field strength
HE	Hydroethidine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGMS	high gradient magnetic separation
HRP- 2	histidine rich protein 2
K_2HPO_4	dipotassium phosphate
$M_{1/2/3}$	marker 1/2/3
M_a	magnetization of wire

MHC	major histocompatibility complex
m_{M1}	geometric mean of marker 1
m_{M2}	geometric mean of marker 2
M_p	magnetisation of particle
m_{rel}	geometric mean quotient
n	number
NaCl	sodium chloride
n_{M2}	number of cells within marker 2
n_{M3}	number of cells within marker 3
O	oxygen
p	parasitaemia
PBS	phosphate buffered saline
PCM	pigment containing monocyte
PMN	polymorphonuclear leukocyte
PfEMP1	*Plasmodium falciparum infected erythrocyte membrane protein 1*
p_{ring}	parasitaemia of synchronized culture in ring-stage
$p_{schizont}$	parasitaemia of synchronized culture in schizont stage
r	radius
RBC	red blood cell
r_{inf}	reinfection rate
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
S.D.	standard deviation
SSC	sideward scatter
v	velocity
v (r)	velocity of particle
V_p	volume of particle
WBC	white blood cell
η	viscosity
π	constant pi
ρ_f	density of fluid
ρ_p	density of particle
“	inch

1 Introduction

1.1 Malaria

Malaria is an infectious disease caused by the eukaryotic parasite *Plasmodium*. It continues to be one of the largest health threats worldwide. More than 2.5 billion persons are at risk (Guerra, Snow & Hay, 2006). Figure 1 shows a map of malaria regions. Between 350 and 500 million people fall ill every year and the disease claims more than one million deaths (Korenromp et al., 2005). Sub-Saharan Africa is the region with the most infections. 90 % of the deaths occur there, mainly in children under the age of five and pregnant women. There are regions with more infections per year than inhabitants living there, hence many people fall ill several times a year (Sachs & Malaney, 2002).

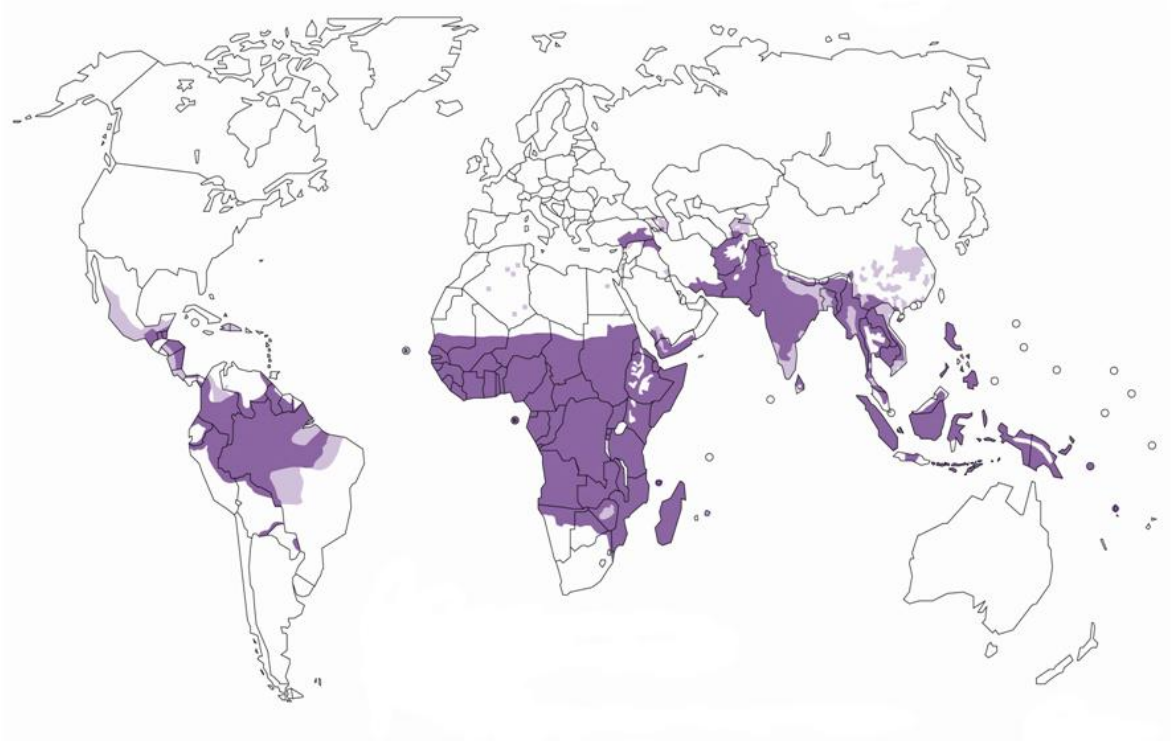

Figure 1

Malaria risk areas
Worldwide map of malaria risk areas in 2006: Dark purple shows areas where malaria transmission occurs and light purple areas are areas with limited risk of malaria transmission (Figure taken from World Health Organization, 2006 [1]).

The word “malaria” has its roots in the Italian expression for “bad air”. For a long time, people believed that the disease was caused by gases of swamps. Malaria was already described millenniums ago. Homer mentions it in his works, for example, and Hippocrates also describes the disease. There are hints that several historical events were influenced by malaria: Alexander the Great’s army suffered from a malaria epidemic when it entered Mesopotamia. Eventually, Alexander himself may have died from it. Ancient Rome was built in the middle of swamps. Its inhabitants had developed some immunity to the disease, but several armies had to break off their campaigns due to malaria epidemics occurring when they approached the city (Schimitschek & Werner, 1985). In World War II, malaria caused many deaths during the construction of the railway tracks through Thailand and Burma. The Japanese took Asian forced labourers and allied prisoners of war and forced them to build the “death railway”. Historians believe that more than 150 000 Asians and 15 000 allied prisoners died. This equals half of the number of all forced labourers. The high mortality rate was due to unbearable health conditions caused by malnutrition, lack of hygiene, and infectious mosquitoes (Fisher, 1947).

1.1.1 Epidemiology and Economic Impact

Researchers have found a strong correlation between malaria and poverty in several regions worldwide. The most representative parameter compared was the per-capita gross domestic product, which were five times less in countries with than in those without malaria. There are different approaches to explain this correlation. One possibility is that malaria promotes poverty. Economic growth increased significantly in countries that managed to eradicate infectious mosquitoes. Children under the age of five are the group in which malaria death occurs most often. High infant death rates correlate with high fertility rates as parents try to compensate for their loss, and high fertility rates in turn promote poverty. Frequent disease also profoundly affects people’s daily lives. Sick children, for example, cannot attend school. Therefore, malaria is an important factor that promotes illiteracy. The survival of malaria sometimes leads to physical as well as mental long term impairment. How many children are affected by mental impairment that can be hardly noticeable remains unclear.

Another possible explanation is that poverty promotes malaria transmission. For example, poor people often sell their life-saving bed nets to obtain money for food (Sachs & Malaney, 2002).

1.1.2 Clinical Manifestation and Diagnosis

The clinical manifestation of malaria is correlated to parasite development within the body. There are no symptoms between the time of infection by a mosquito bite and the release of thousands of merozoites that have developed within infected liver cells. The merozoites enter and develop within red blood cells (RBCs) for several cycles. Each cycle ends with the rupture of the infected RBCs. The onset of the disease is marked by symptoms of a mild flu, and host inflammatory reactions and anaemia then follow. The characteristic fever episodes are likely caused by inflammatory cytokine production induced by the parasite. As the speed of intraerythrocytic development is species-specific, these intervals have different durations. This is important for the differential diagnosis. In case of *Plasmodium falciparum,* the fever episodes often display no periodicity. High numbers of RBCs can become infected within a relatively small number of cycles. Their destruction causes anaemia, which can become so severe that the oxygen supply of cells cannot be maintained. This leads to cell hypoxia and death.

Malaria tropica caused by *Plasmodium falciparum* can have life-threatening complications. The parasite produces proteins during its intraerythrocytic development, e.g. *PfEMP1 (Plasmodium falciparum infected erythrocyte membrane protein 1)*. These are exported and inserted into the host cell membrane and present as knob-like structures as visualized in the electron microscope. Parasitized RBCs bind to the endothelia of small vessels. This sequestration, can occur in various organs and most often manifests in the brain, the lung, and the kidney causing cerebral malaria, ARDS (acute respiratory distress syndrome), and kidney failure. These complications are the hallmark of severe malaria and underlie the lethal potential of the disease (Lang & Löscher, 2000).

To this day microscopic examination of blood smears remains the most important tool in diagnosis of malaria. Thick blood films are instrumental for primary detection of infected RBCs, while thin blood films offer the possibility to quantify the parasitaemia and determine the *Plasmodium* strain. Parasitaemia can also be estimated in thick blood films through counting parasitized RBCs per 200 white blood cells (WBCs) and multiplying this number by 40. Microscopic examinations are cheap and only require a light-microscope. However, they are time-consuming and there is a demand for other tools. A rapid test for *Plasmodium falciparum* based on the detection of membrane protein *HRP-2 (histidin-rich-protein-2)* is already in use (Warhurst & Williams, 1996). Other methods are in development, but they generally lack sensitivity and/or specificity. Some also require

expensive equipment like a flow cytometer, which is not broadly available, especially in the poor endemic regions worldwide.

1.1.3 Prevention and Treatment

The best epidemiological control measure for malaria is prevention through the use of impregnated bed-nets, appropriate clothing and effective insect repellents.

The alternative approach of vector control has been disappointingly unsuccessful. A worldwide campaign against malaria was initiated with the introduction of the pesticide DDT (dichlorodiphenyltrichloroethane) in the 1950s. Unfortunately, this resulted only in the eradication of vectors in temperate zones, due to lack of accuracy in the highly endemic, mostly poor areas and to growing parasite resistance to DDT (Greenwood & Mutabingwa, 2002).

Quinine was the first widely used medication for the treatment of malaria. The agent inhibits formation of malaria pigment, which leads to parasite death within the infected RBC. The drug is very effective, but unfortunately has many side effects. Newer drugs were introduced in the middle of the last century that have gradually replaced quinine. A breakthrough was the discovery of chloroquine, a drug with the same mechanism of action but with much reduced toxicity (Lang & Löscher, 2000). Following its introduction and the successful vector eradication in temperate zones, there was a loss of interest in drug development. From 1975 to 1996 there were only three new antimalarial drugs introduced worldwide. This has led to the growing resistance of parasites, especially towards chloroquine. Today, there are regions where nearly all *Plasmodium falciparum* strains are resistant to this agent. The interim loss of interest created a peculiar situation: today's possibilities to cure malaria are worse than decades ago. In recent years, the worldwide burden of the disease has regained recognition and research efforts have intensified (Greenwood & Mutabingwa, 2002).

1.1.4 Immunity and Vaccine Development

When an individual is repeatedly exposed to *Plasmodia*, the body is capable of mounting an immune response that leads to semi-immunity. This does not prevent, but reduce the severity of the disease. Semi-immunity is lost within months when a person moves away from a malaria region. Furthermore, semi-immunity does not cross-protect against different *Plasmodium* species which can vary within just a few kilometres.

The constant exposure of humans to malaria has led to development of natural immunity by genetic variation. Again, the disease is not totally prevented, but the genetic variations lead to improved survival (Bogitsh, Carter & Oeltmann, 2005). The classic example is sickle-cell anaemia. This monoclonal inheritance leads to better survival rates in heterozygous persons. Homozygous individuals do not exist, as a homozygous gene combination leads to abortion of the foetus. In spite of this selection disadvantage, it is a widely spread inheritance in Africa where the burden of malaria is so strong (Sachs & Malaney, 2002).

There are three main approaches to development of a malaria vaccine: the anti-sporozoite vaccines, the anti-asexual blood stage vaccines, and the transmission-blocking vaccines. However, *Plasmodia* are eukaryotic cells and can mutate and adapt to multiple conditions. There is lack of a vaccination to totally prevent malaria, but some vaccines appear to increase the survival rate of children under the age of five. The genome of *Plasmodium* has been sequenced, which might lead to new approaches in drug as well as vaccine development (Bogitsh, Carter & Oeltmann, 2005).

1.2 Plasmodium Falciparum

Plasmodium belongs to the *Phylum* of *Apicomplexa* (syn. *Sporozoa*). It is a monocellular eukaryote (*Protozoa*). There are more than 50 different species of *Plasmodia* (Bogitsh, Carter & Oeltmann, 2005). Four of these are pathogenic for humans: *Plasmodium falciparum* causing *malaria tropica*, *Plasmodium vivax* and *Plasmodium ovale* causing *malaria tertiana,* and *Plasmodium malaria* causing *malaria quartana*. This work focuses on *Plasmodium falciparum*, which is the most dangerous species that causes life-threatening disease. (Lang & Löscher, 2000).

1.2.1 Life Cycle

The life cycle of *Plasmodia* involves two different hosts, one being a female mosquito of the *Genus Anopheles*, the other being a vertebrate. Mosquitoes of the species *Anopheles gambiae* are a special health threat because of their high anthropophily. The part of the cycle within the mosquito starts when the insect has taken up female macrogametocytes as well as male microgametocytes by biting one or several infected vertebrates, in whom these sexual forms have developed. Sporozoites develop within the mosquito and invade the salivary gland. Whenever the mosquito bites a vertebrate it releases those sporozoites into the bloodstream. In case of the vertebrate being susceptible, the further life cycle can take place.

With help of their A*picomplexa*, which gave this *Phylum* its name, the sporozoites can enter into the hepatic cells. The exoerythrocytic schizogony takes place within the next 5 to 15 days, leading to preerythrocytic schizonts. 10 000 to 30 000 merozoites are released upon cell rupture into the bloodstream and invade RBCs, and erythrocytic schizogony begins (Sturm et al., 2006). The parasites grow within the RBCs and develop from rings to trophocytes and finally to schizonts. The schizonts generate new merozoites that are released into the bloodstream when the cells rupture. The intrinsically short-lived merozoites must reinvade other RBCs within 30 seconds. The asexual erythrocytic life cycle is visualized in Figure 2. After host cell entry, some merozoites develop into one of the sexual life forms mentioned above. Triggers for this alternative development are still unknown. Uptake of gametocytes by a mosquito initiates a new life cycle (Fujioka & Aikawa, 2002).

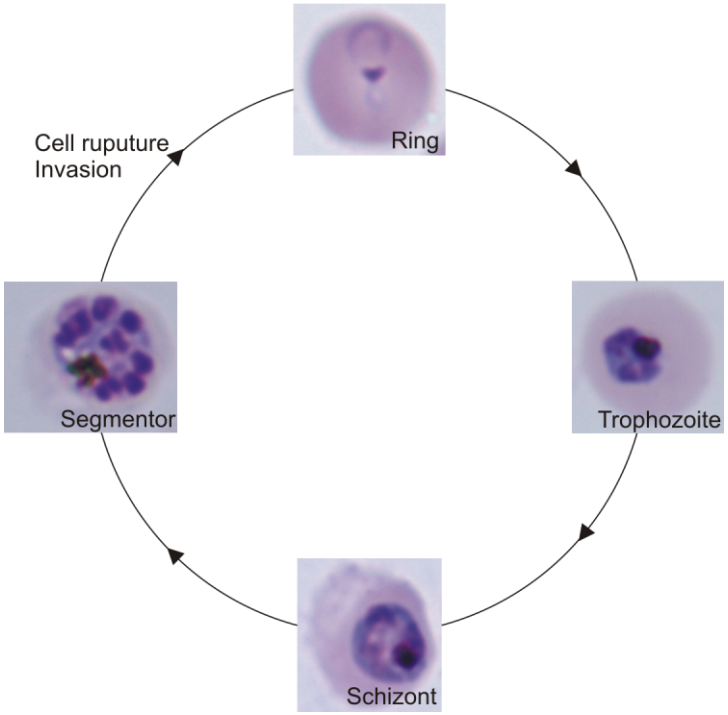

Figure 2

Erythrocytic asexual life cycle of *Plasmodium*
The erythrocytic life cycle begins after merozoites released from infected liver cells invade RBCs. The parasites then infect and develop within the RBCs. The parasites first develop into ring-stage, followed by trophozoite and schizont stage. In the last hours of development the schizonts are also called segmentors due to the already visible future merozoites. Finally the host RBCs rupture. The cycle is closed with invasion of merozoites into other RBCs.

1.2.2 Ultrastructure of Erythrocytic Stages

The lemon-shaped merozoites released from a ruptured RBC are very small. They have a width of 1 µm and a length of 1.6 µm. They have organelles typical of eukaryotic cells: a nucleus, one mitochondrion, ribosomes, and cytoskeletal components. Moreover, they have a three layer membrane and a plastid. This is an organelle characteristic of *Apicomplexa*. In plant cells, it is the locus of photosynthesis. Special organelles, together forming the apical complex, facilitate entry to the new host RBC: rhoptries, micronemes, and dense granules. The parasite binds to special antigens on the RBC membrane (Bannister et al., 2000). *Plasmodium falciparum* binds to Glycophorin A, which is present on all RBCs. Theoretically, all RBCs of the host can therefore be infected. This is in contrast to other species, where host cell entry is limited to antigens that are expressed on a fraction of the RBCs only (Lang & Löscher, 2000).

In the first hours after host cell entry the parasite is referred to as ring-stage. This is due to its signet-ring like appearance under the light microscope when it is stained. The ultrastructural correlate is a thick rim of cytoplasm, containing the major organelles and the

nucleus. The nucleus appears as the signet of the ring. The parasite can have different possible configurations: discoid, flat, or cup-shaped. Through a ring structure called the cytosome, small portions of RBC cytoplasm are ingested into small vesicles of the parasitophorous vacuole membrane. Haemoglobin degradation starts. The parasite utilises some of the released amino acids. Haemozoin, the malaria pigment that results from heme detoxification, will be described in more detail below. It is stored in several small pigment vacuoles. During their development, these vacuoles fuse to form a single large pigment vacuole.

The shape of the parasite changes with its growth. It becomes rounder and more irregular, and is referred to as the trophozoite. Figure 3 shows an example. The configuration change implies enlargement of the parasite membranes. Moreover, the cytoplasm and the membrane of the RBC are gradually altered. Mechanisms underlying movement of molecules between parasite and host compartments are currently under intensive study (Lingelbach & Przyborski, 2006).

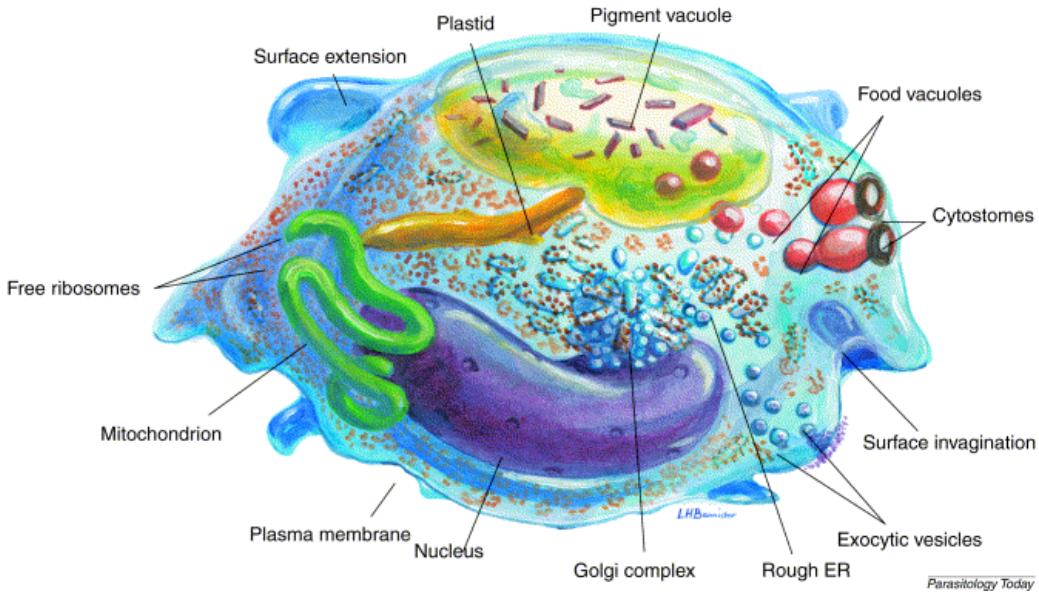

Figure 3

Mid-trophozoite stage of *Plasmodium falciparum*
This stage is characterized by its irregular outline, the increase in protein synthesizing apparatus, growth of the pigment vacuole and structures associated with export of parasite proteins (Bannister et al., 2000).

The last intraerythrocytic stage is referred to as the schizont stage. It is dominated by merozoite formation and shown in Figure 4. It begins with repetitive endomitotic nuclear division and multiple cytoplasmatic changes. Then formation of 16 to 20 new merozoites

takes place at foci around the circumference of the parasite. Throughout this stage haemoglobin degradation and haemozoin formation persist. Finally, merozoites are released into the blood stream by cell rupture (Bannister et al., 2000).

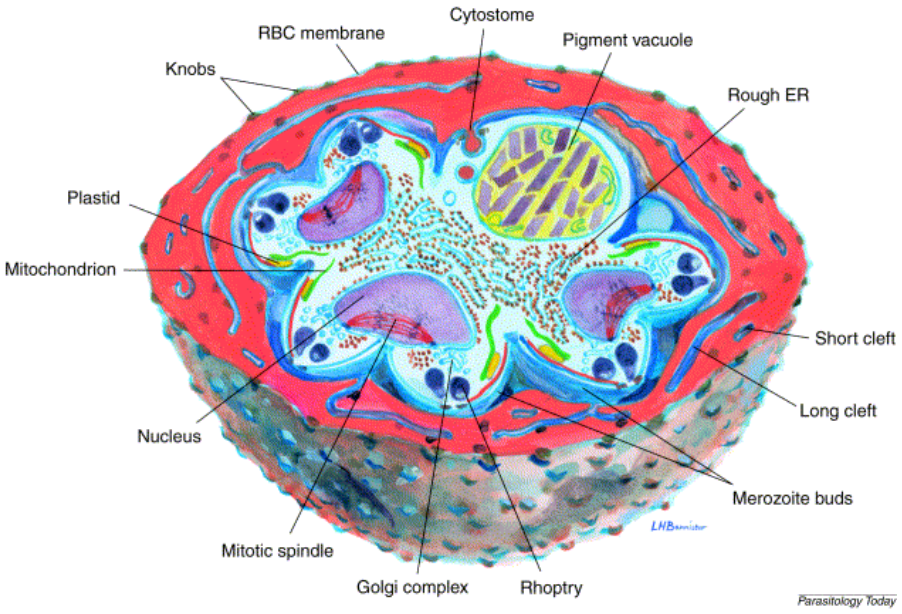

Figure 4

Schizont stage of *Plasmodium falciparum*
Organization of a *Plasmodium falciparum* schizont towards the end of that stage, depicted in a schizont infected RBC cut transversely to show parasite and RBC structure. Surface knobs can be seen (Bannister et al., 2000).

1.2.3 Haemozoin- the Malaria Pigment

As early as in the 17th century, there were descriptions of discolorations in the internal organs of malaria victims (Egan, 2002). In 1847, the psychiatrist Meckel described black-brown pigment granules observed in an insane patient (Meckel, 1847). But it was Virchow in 1849, who first linked the pigment to malaria. In following decades, it was of crucial importance in the identification of the parasite and in malaria research (Hempelmann, 2007).

Haemozoin, a pigment formed of polymerized, degraded haemoglobin, develops during the intraerythrocytic life cycle. The pigment is clearly visible under the light microscope from trophocytic stages onward. It is released into the blood stream when infected RBCs rupture. The body is not able to recycle the iron atoms of haemozoin. This leads to iron deficiency and the suppression of erythropoesis, important factors leading to anaemia. The haemozoin is deposited in the organs of the body, which explains the discolorations described centuries ago. It can also be seen in WBCs that have ingested it and long term

experiments have shown that it accumulates in the spleen (Hänscheid, Egan & Grobusch, 2007).

Despite intensive research in recent decades, many molecular aspects underlying pigment formation have remained unresolved. Ultrastructural studies have located haemozoin to the food vacuole of the parasite. Biochemical analyses revealed that it represents a polymerization product of heme groups, consisting of many heme-dimers that are covalently bounded to each other (Egan, 2002).

Haemozoin formation represents a detoxifying step of fundamental importance to the survival of the parasite. Degradation of haemoglobin leads to the release of free heme with a central iron in the state of Fe^{3+}, which is lipophilic and can intercalate with cell membranes, potentially leading to cell lysis. The parasite solves this problem by detoxifying haemozoin through polymerization (Kumar et al., 2007).

This leads to the question why the parasite degrades haemoglobin in the first place. Until recently, it was generally assumed that the parasite does so in order to utilize the amino acids. However, there have been findings showing that the parasite uses only small amounts of the amino acids derived from haemoglobin (Krugliak, Zhang & Ginsburg, 2002). An intriguing explanation for haemoglobin degradation might be that consumption of haemoglobin could be crucial for the host cell's osmotic stability towards the end of the parasite's intracellular life cycle, a speculation that currently warrants further research (Lew, Tiffert & Ginsburg, 2003).

One characteristic of haemozoin is of special importance for the methods we will introduce: it is paramagnetic and a paramagnetic particle displays magnetic susceptibility. Magnetic susceptibility is defined as the degree of magnetisation of a material in a magnetic field. The equation

$$J = \chi H$$

describes this. J is the magnetisation of the particle, H is the magnetic field strength and χ is the magnetic susceptibility. χ is dimensionless, as J and H both have the dimension Am^{-1} (Meschede, 2006).

A haemoglobin molecule consists of globin chains and four heme groups, each carrying an iron atom in the state of Fe^{2+} in its centre. The globin chains are and remain non-susceptible to magnetic fields. So the focus needs to be set on the heme groups. The heme groups in oxygenated haemoglobin do not have any ionic bonds and therefore the whole particle is non-susceptible.

Chemical bonds between the iron atom and the rest of the heme group change with deoxygenation of the haemoglobin, resulting in four unpaired electrons per heme group. These ionic bonds render the haemoglobin paramagnetic. A fully deoxygenated RBC in aqueous suspension has the magnetic susceptibility of 0.265×10^{-6} (Zborowski et al., 2003).

In haemozoin the heme group is oxidized and the iron atom is in the state of Fe^{3+}, resulting in five unpaired electrons per heme group. Again the ionic bonds render haemozoin paramagnetic. The susceptibility of an infected RBC increases proportionally to haemozoin formation. There have been findings that susceptibility increases gradually throughout the whole intraerythrocytic cycle of *Plasmodium falciparum* (Moore et al., 2006). Therefore no certain number for magnetic susceptibility can be given.

Methaemoglobin also consists of oxidized heme groups with an iron atom in the state of Fe^{3+} and carries five unpaired electrons per heme group. This renders it paramagnetic. A fully oxidized RBC in aqueous suspension has the magnetic susceptibility of 0.301×10^{-6} (Zborowski et al., 2003). However, this is a theoretical finding, since fully oxidized RBCs do not occur in blood cultures. Oxidation of haemoglobin does constantly happen within RBCs. Yet, the cells maintain two methaemoglobin reducing pathways to reverse this event (Tomoda et al., 1980). On average the fraction of oxidized haemoglobin does not exceed 1 % and can be neglected in the experiments to be observed.

In short, RBCs can or cannot display magnetic susceptibility. An oxygenated RBC does not display any susceptibility. In contrast, deoxygenated RBCs display susceptibility and this increases when haemoglobin is oxidized. Parasitized RBCs display magnetic susceptibility that increases proportionally with haemozoin content.

This will be exploited in the methods described below. Cells are first fully oxygenated, so that paramagnetic property is solely determined by haemozoin. This forms the basis for the application of HGMS to cells infected with *Plasmodium falciparum*.

1.3 High Gradient Magnetic Separation (HGMS)

HGMS is a widely applied separation technique. It functions on the basis of magnetic forces and allows the separation of weakly magnetic particles from non-magnetic ones that cannot be separated by conventional magnetic separators. This technique was introduced in the late sixties of the last century and has found multiple fields of application, e.g. purification in steel and mineral processing industries (Meisel, 2004). It has also found many applications in the biophysical field.

1.3.1 Theoretical Background

A paramagnetic particle in suspension passing a magnetic field will have several forces acting on it. A paramagnetic particle is a particle with a permanent magnetic dipole moment but without any preferred direction. The resulting force is F_p:

$$\vec{F}_p = \vec{F}_m + \vec{F}_d + \vec{F}_g .$$

F_m represents the magnetic force, F_d the drag force of the fluid and F_g the buoyant force (for better legibility vectors will only be written in formulas).

The magnetic force F_m acting on a particle can be generally described as

$$\vec{F}_m = V_p\left(\vec{M}_p\vec{\nabla}\right)\vec{H} .$$

V_p is the volume and M_p the magnetization of the particle. H represents the magnetic field strength. The equation shows that F_m increases with the magnetic field gradient. Since the non-permanent magnetization M_p is proportional to H, the magnetic force is proportional to the magnetic field strength, too.

The drag force is given by Stokes' law

$$\vec{F}_d = 6\pi\eta\frac{d}{2}\left[\vec{v}(\vec{r}) - \frac{d\vec{r}}{dt}\right]$$

η stands for the viscosity of the fluid and dr/dt stands for its velocity. d is the diameter of the particle and $v(r)$ its velocity. This shows that the drag force increases with viscosity, particle size and velocity difference of fluid and particle.

The buoyant force is given by

$$\vec{F}_g = \rho_p V_p\left(1 - \frac{\rho_f}{\rho_p}\right)\vec{g}$$

ρ_p and ρ_f represent the densities of particle and fluid. g is gravity and V_p is the volume of the particle, as above. This shows that the buoyant force depends on the density difference of particle and fluid (Schewe, Takayasua & Friedlaender, 1980).

A possible setting to reach separation of particles consists of a fluid passing a column, where the magnetic field is directed transversally to the fluid velocity, whereas the drag force and the buoyant force both are directed vertically. This is shown in Figure 5. Particles will stay within the column, if F_m is greater than the competing forces F_d and F_g. This will allow separation of particles, as they stay within the column while non-magnetic components flow through. The degree to which the force F_m dominates the perpendicular forces, determines the efficiency of particle capture.

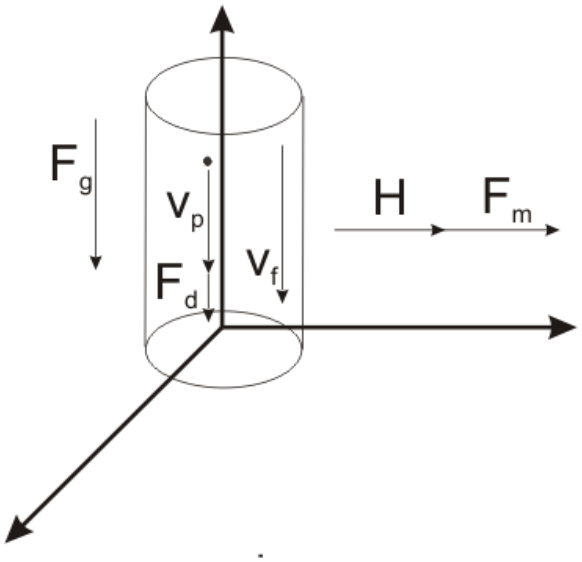

Figure 5

Direction of forces
The figure shows how forces need to be directed to have a possible setting for magnetic separation.

One possibility to increase F_m is to increase the magnetic field gradient. This is reached by inserting small sized ferromagnetic elements like steel wool wire into the magnetic field. For the distance of approximately the diameter of the wire a, this results in a high gradient magnetic field around the wire. The magnetic field at a point very near to the wire surface can be described as:

$H_0 + 2\pi M_a$.

After the distance a, the magnetic field equals H_0. Only particles with a diameter similar or smaller than a can be caught. The gradient within the field around the wire can be

described as M_a/a. This gradient behaves inversely proportional to the diameter of the wire. Thus very small diameters will result in high gradient magnetic fields (Oberteuffer, 1973). There are three possible settings of spatial arrangement of the wire, to reach maximum high magnetic field gradients. They are commonly described as the transverse (a), longitudal (b) and axial (c) case and shown in Figure 6 (Schewe, Takayasua & Friedlaender, 1980).

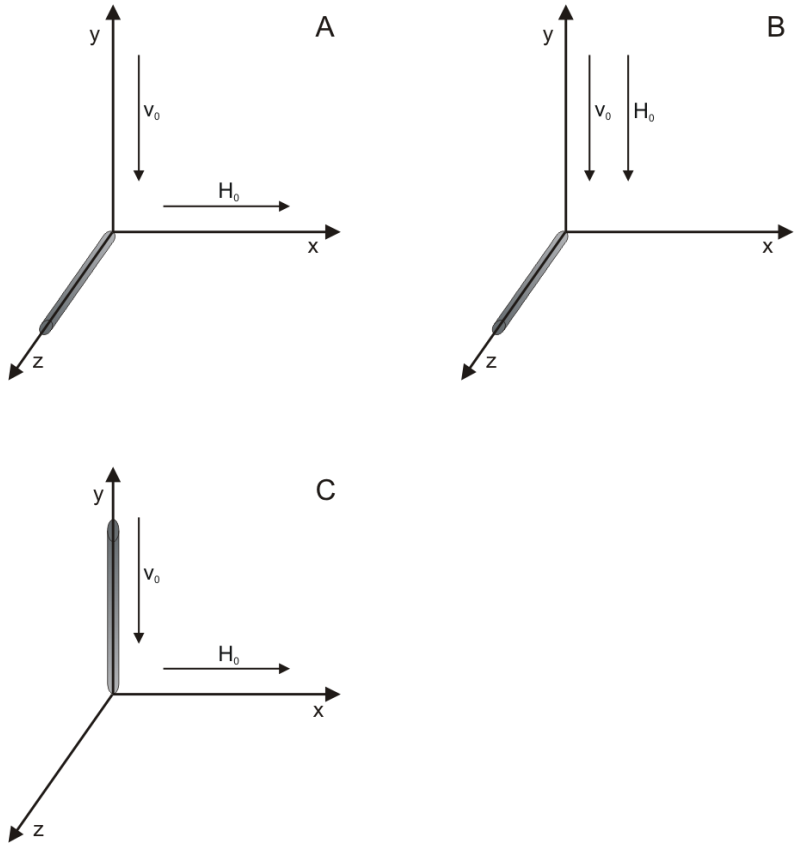

Figure 6

Geometrical configurations for magnetic separation
Representation of the three geometrical configurations: (A) shows transverse, (B) longitudal, and (C) axial configuration of the wire within the magnetic field (Figure on basis of Schewe, Takayasua & Friedlaender, 1980).

1.3.2 Design of an HGMS Column

According to this theoretical background a general construction of an HGMS column must consist of the following components: First, a strong magnetic field, which can be given by a rare earth dipole magnet. It also can be generated by an electromagnet. Second, a column containing a ferromagnetic matrix needs to be within this magnetic field. The column forms a channel for the fluid and the matrix generates the high magnetic field gradients. The column needs an opening at the beginning and at the end for inlet and outlet of the fluid containing the paramagnetic components that shall be captured. A flow regulator at

the outlet allows varying flow velocity and interrupting flow. Another helpful component is a reservoir on the top of the inlet, containing fluid. A schematic setting is shown in Figure 7.

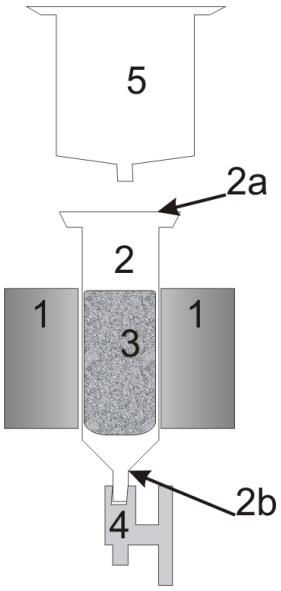

Figure 7

Schematic drawing of an HGMS column
(1) shows the dipole magnet generating a high magnetic field, (2) column with (2a) inlet and (2b) outlet, (3) ferromagnetic matrix that generates the high gradients within the dipoles of the magnet (1), (4) flow regulator put on the outlet and (5) optional reservoir.

In general, there are two different ways to use an HGMS column. The first is cell purification. For this application, the particles captured within the column are collected. To achieve this, the column is washed after loading, the magnetic field is then removed and the adherent cells are harvested. Flushing can be done at higher velocities, to increase the removal drag force (Owen, 1978). Application of this technique for malaria cultures leads to purification of late-stage infected, magnetically susceptible RBCs.

The other possible application is particle separation by depletion. This is the way it is commonly used in industrial processes. In this case, a fluid is passed through the column and paramagnetic particles are captured within it. The purified fluid, which has been depleted of paramagnetic particles, is collected when it leaves the column (Oberteuffer, 1973). Application of this technique for malaria cultures leads to depletion of late-stage infected, magnetically susceptible RBCs. The fluid collected will contain non-infected, oxygenized RBCs and ring-stage infected, not yet paramagnetically susceptible RBCs.

1.3.3 HGMS for Cell Separation

HGMS was applied for cell separation soon after its first descriptions, as highly purified cells are often required for cell culture based research. In the beginning, cells that intrinsically showed magnetic spins were separated. Early examples are RBC separation from full blood (Melville, 1975) and separation of paramagnetic oxidized cells from diamagnetic oxygenized cells (Owen, 1978). Cell separation on the basis of HGMS has remained an issue of research throughout recent decades. Its application has been taken beyond cells that can be intrinsically paramagnetic by introducing paramagnetic antibodies. Target cells are labelled and separated by antibodies that have a paramagnetic bead linked to their Fc region (Kemshead & Ugelstad, 1985). Often, these separations are done with commercially available polymer coated columns (Miltenyi et al., 1990). Nowadays, HGMS is a standard method in culture based research (Stewart et al., 2000) and new applications are frequently described. For example, a magnetophoretic microseparator has been introduced for efficient separation of RBCs and WBCs from whole blood (Han & Frazier, 2006).

1.3.4 HGMS for RBC Separation

As outlined above, RBC separation started with exploitation of their intrinsic variability of paramagnetic spin. Both Melville (Melville, 1975) and Owen (Owen, 1978) reported that columns had to be packed with stainless steel wool, as cell lysis was observed with ordinary steel wool. Otherwise separation works with permanent as well as electric magnets. Permanent magnets are easier to handle: they require less space and work without electricity.

In 1981, Paul was the first to describe purification of malaria infected RBCs. This procedure allowed approximately 40- fold concentration of the cells. He published two articles where purification was described for *Plasmodium berghei* parasitized mouse red blood cells (Paul et al., 1981a), as well as blood cultures infected with *Plasmodium falciparum* (Paul et al., 1981b). In the first article he described that trophozoites and schizonts were purified to a concentration of 0.527 % from a starting concentration of 0.0125 % in a one column run. In the other publication *Plasmodium falciparum* parasitized cells were obtained at a purety of 83 to 92 % from a starting parasitaemia of 0.1 to 4.5 %. However, this could only be achieved through performance of several column runs. These results showed applicability of HGMS purification for infected RBCs, but results were not good enough to find application in basic research.

Paul published another study four years later that described the viability of RBCs and WBCs after passing through an HGMS column (Paul, Melville & Roath, 1985). *In vivo* comparison in mice showed no differences between *Plasmodium berghei* infected RBCs that had or had not been exposed to highly magnetic forces respectively (Trang et al., 2004). However, in general, biological effects of magnetic fields are a field of research discussed controversially (Grissom, 1995). Due to that, there is some reservation remaining regarding the potential utility of HGMS.

After these first descriptions, many years passed without any reports on HGMS based isolation of parasitized RBCs, but the theme has regained interest in recent years. In 2004, Trang et al. described one step concentration and purification of *Plasmodium berghei* from freshly drawn whole blood from mice (Trang et al., 2004). In the best experiments, purification of infected RBCs up to 98 % was achieved. Stage-specific analysis of the parasitized cells was not given. Last year, HGMS-based purification for all human *Plasmodium* species was described (Ribaut et al., 2008). The level of purification depended on the malaria strain and ranged between 90 to 98 %. However, a prerequisite was that cultures had to first be synchronized by sorbitol lysis before the HGMS step.

1.4 Synchronization

The introduction of the continuous culture of *Plasmodium falciparum* had a tremendous impact on the possibilities of research and opened new avenues to long-term investigations into the cell biology of the parasite (Trager & Jensen, 1976, Trager & Jensen, 1997). Research possibilities were further boosted through the advent of synchronized cultures. Synchronized cultures are widely applied to study the mechanisms of drug resistance (Srinivas & Puri, 2002), parasite multiplication (Inselburg & Banyal, 1984; Gritzmacher & Reese, 1984), and they are also invaluable as tools to establish new diagnostic procedures (Jouin et al., 2004).

The easiest and most commonly used method of synchronization is the procedure developed by Lambros and Vanderberg (Lambros & Vanderberg, 1979). In this method, RBCs infected with late-stage parasites are selectively lysed by exposure to isoosmolar 5 % sorbitol solution. RBCs infected with ring-stage parasites remain intact, resulting in a culture with ring-stage parasitized cells only. Lambros and Vanderberg realized that the isoosmolar 5 % sorbitol solution has an osmolality of 287 mOs/kg which differs only slightly from that of the culture medium (276 mOs/kg). Selective lysis of late-stage infected cells appears to stem from differences in RBC membrane permeability for sorbitol due to insertion of new permeability pathways in parasitized RBCs. Sorbitol enters late-stage infected cells and causes cell lysis (Kutner et al., 1985).

Another widely applied method is synchronization and purification of late-stage infected erythrocytes by Percoll density gradient centrifugation. Density of parasitized RBCs decreases with parasite maturation (Nillni, Londner & Spira, 1981). Whenever certain fractions of parasitized cells are needed, separation can be carried out with hypertonic, discontinuous Percoll-sorbitol gradients. Hypertonicity causes a cell shrinkage, but infected cells swell back due to an influx of sorbitol through the new permeability pathways. This increases the density gaps between the different developmental stages and allows better separation in the Percoll gradients (Kutner et al., 1985). In contrast to the method of Lambros and Vanderberg (Lambros & Vanderberg, 1979), swelling does not cause cell lysis.

Knob expressing strains of *Plasmodium falciparum* can also be synchronized by suspending cells in a gelatine solution. Gelatine induces rouleaux forming of RBCs, which increases their sedimentation rate. Late-stage infected RBCs show a different

sedimentation behaviour and can therefore be separated (Jensen, 1978; Lelievre, Berry & Benoit-Vical, 2005).

All described methods do not achieve a totally synchronized culture. Age spans are wide and contamination with parasites of other stages often disrupts synchronization within a short time. In publications where a high accuracy of synchronization is needed, different methods are combined or repeated to achieve highly purified cultures with small age spans (Inselburg & Banyal, 1984). Lambros and Vanderberg (Lambros & Vanderberg, 1979) suggested a "fine tuning" by repeating the sorbitol lysis after 34 h, a procedure that has found increasing application (Schuster, 2002).

All available methods do not reach synchronicity by influencing the parasites to develop at the same speed, but by providing a culture, where all parasitized cells are at the same stage of development.

1.5 Flow Cytometric Analysis of Parasitized Cells

Flow cytometry is a technique that allows qualitative and quantitative analysis of cells in suspension. Cells in suspension are passed in laminar flow through a 50 to 100 µm capillary. A laser is focused on the centre of the capillary. The volume of the cell leads to a small angle of distraction measured in the forward scatter (FSC). Cell granulation leads to a wide angle of distraction measured in the sideward scatter (SSC). In addition to these physical parameters, multiple fluorescence signals can be measured (Radbruch, 2000).

Flow cytometry is an important tool in malaria research that has been widely used in the last decades. It has contributed to the development of new procedures for malaria diagnosis (Bucana, Saiki & Nayar, 1986; Grobusch et al., 2003). There are different dyes that allow stage specific analysis. Those used in this thesis will be introduced in the following section.

1.5.1 Stage Specific Analysis of Infected RBCs

Stage specific analysis of infected RBCs can be achieved by using the dye Acridine Orange (AO). This is a weak base that binds to cellular poly-anions, mainly staining DNA, RNA and also accumulating in the very acidic food vacuole of the malaria parasite. Binding to double strand nucleic acids leads to the emission of green fluorescence with a peak around 532 nm excited by blue light of 488 nm, whereas single strand nucleic acids emit a red fluorescence with a peak around 650 nm (Bauer & Dethlefsen, 1980). During cell cycle development most of the DNA forms double strands, whereas RNA mostly forms single strands. Hare and Bahler (Hare & Bahler, 1986) successfully applied the staining for stage specific analysis of *Plasmodium falciparum* cultures. Recently, an improved protocol that does not require fixation of cells was introduced (Bhakdi et al., 2007). Analysis in a dot plot with AO-red on the x-axis and AO-green on the y-axis gives a characteristic picture of a culture of *Plasmodium falciparum*, in which different populations of cells can be discriminated. An example is given in Figure 8. Increases in DNA and RNA accompanying development of the parasites in the RBC, correlate with increase in fluorescence. This offers the possibility to analyse the success of the depletion of late-stage infected cells from a culture.

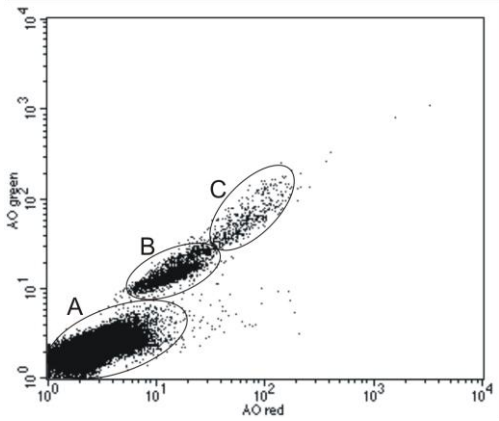

Figure 8

Flow cytometric analysis of a *Plasmodium falciparum* cell culture stained with AO
The first population (A) represents non-infected RBCs. In the given dot plot 93.3 % of the counts are within region (A). The other populations (B) and (C) represent infected RBCs. The population (B) with low fluorescence represents early stages and (C) with high fluorescence represents late stages of infection.

Another dye that allows stage specific analysis of infected RBCs is Hydroethidine (HE), a reduced form of ethidium bromide. It shows the characteristics of an uncharged, racematic compound and is widely used as a vital dye for flow cytometry. The mechanism of staining seems to rely on the intracellular dehydrogenation of HE to ethidium that causes binding to DNA (Bucana, Saiki & Nayar, 1986). Further studies found a correlation between increasing DNA content during parasite growth and increase of fluorescence of the parasitized RBCs, and the staining method has found application in stage specific cytometric analysis of cultures infected with *Plasmodium falciparum* (Pattanapanyasat et al., 2003; van der Heyde et al., 1995). The procedure was also employed in some of the experiments of this thesis.

2 Objective of the Thesis

This thesis was undertaken to explore possible applications of high gradient magnetic separation (HGMS) for the capture of erythrocytes infected with *Plasmodium falciparum*, the aims being to develop novel methods for purifying late-stage infected cells, for selectively depleting cell cultures of the latter, and for establishing synchronized cell cultures.

3 Materials

3.1.1 Equipment

- Centrifuge Allegra X-15 R (Beckman Coulter; Fullerton, CA)
- CO_2-Incubator (Sanyo; Moriguchi, Japan)
- Dipole magnet with two plates 50x30x12 mm; 1,29 T (Neotexx; Berlin, Germany)
- Flow cytometer FACS-Calibur (Becton-Dickinson; Erembodegem, Belgium)
- Fridge (Sanyo; Sakata, Japan)
- Micro haematocrit centrifuge (Damon IEC; Milford, MA)
- Micro-centrifuge Spectrafuge 16 M (Labnet; Edison, NJ)
- Microscope Axiostar plus (Zeiss; Jena, Germany)
- Microwave (Sharp, Osaka, Japan)
- Pipet-aid (Drummond; Broomall, PA)
- Scale Adventurer (OHAUS; Pinebrook, NJ)
- Stir bar (Corning; Corning, NY)
- Stirrer/Hotplate (Corning; Corning, NY)
- UVUB 1200 flow hood (Uniflow; Martinsried, Germany)
- Vortex (Scientific Industries; Bohemia, NY)
- Waterbath (Julabo; Seelbach, Germany)

3.1.2 Materials

- 45 µm Filter (Satorius; Göttingen, Germany)
- Acridine Orange (Invitrogen, Molecular Probes; Eugene, OR)
- Albumin, bovine fraction V (Sigma-Aldrich; St. Louis, MO)
- Alcohol, 70% (General Hospital Products; Pathum Thani, Thailand)
- BD FacsClean (Becton-Dickinson; Erembodegem, Belgium)
- BD FacsFlow (Becton-Dickinson; Erembodegem, Belgium)
- BD FacsRinse (Becton-Dickinson; Erembodegem, Belgium)
- BD-Falcon [Polystyrene] (Becton-Dickinson; Erembodegem, Belgium)
- Cover-glass (Sailbrand; Suzhou, China)
- Culture flask; 25 cm^2, 50 cm^2 (Corning; Corning, NY)
- Gelatine from bovine skin type B, 75 bloom (Sigma-Aldrich; St. Louis, MO)
- Giemsa stain (Sigma-Aldrich; St. Louis, MO)

- Glucose (Sigma-Aldrich; St. Louis, MO)
- HEPES-buffer (Sigma-Aldrich; St. Louis, MO)
- Human serum (Bloodbank of Siriraj Hospital; Bangkok, Thailand)
- Hydroethidine (Invitrogen, Molecular Probes; Eugene, OR)
- Improved Neubauer chamber (Boeco; Hamburg, Germany)
- K_2HPO_4 (Sigma-Aldrich; St. Louis, MO)
- Malaria strain TM 267 (Laboratory strain)
- Micro haematocrit tubes (Vitrex Medical A/S; Hervel, Denmark)
- Microscope slides, frosted (Sailbrand; Suzhou, China)
- Phosphate buffered saline (Sigma-Aldrich; St. Louis, MO)
- Pipette; 0.5-10 µl, 2-20 µl, 5-50 µl, 50-200 µl, 200-1000 µl, 1-5 ml (BioHit; Helsinki, Finland)
- Pipette-tip (BioHit; Helsinki, Finland)
- RBCs Group 0 Rh+ (Voluntary donors from laboratory personell)
- RPMI 1640; R6045 (Sigma-Aldrich; St. Louis, MO; St. Louis, MO)
- Single-use needle 20-23 G 1-1.5“ (Nipro; Zaventem, Belgium)
- Single-use syringe; 5, 10, 20, 50 ml (Nipro; Zaventem, Belgium)
- six well culture cluster (Corning; Corning, NY)
- Sodium-bicarbonate (Sigma-Aldrich; St. Louis, MO)
- Stainless steelwool size „fein“ (Oscar Weil GmbH; Lahr, Germany)
- Sterile water (General Hospital Products; Pathum Thani, Thailand)
- Sucrose (Sigma-Aldrich; St. Louis, MO)
- Tube; 15, 50 ml (Corning; Corning, NY)
- Two-way stopcock, three-way stopcock (Bio-Rad; Hercules, CA)

3.1.3 Software

- Cell Quest Pro (Becton-Dickinson; Erembodegem, Belgium)

4 Methods

4.1.1 Malaria Cultures

Plasmodium falciparum laboratory strain TM 267 was cultured as described by Trager and Jensen (Trager & Jensen, 1976). The culture medium was RPMI 1640 containing HEPES 5.94 g/l, glucose 1 g/l, hypoxanthine 50 mg/l, 5 % sodium bicarbonate and 10 % human AB serum. Cultures were kept in an incubator under 5 % CO_2 atmosphere at 37°C and the medium was changed every day at the same time.

Parasite cultures were maintained in cell culture flasks at a haematocrit of about 5 %. 25 cm² culture flasks contained 100 µl packed cells (equivalent to approximately $1x10^9$ cells) in 5 ml of culture medium, and 50 cm² flasks contained 250 µl of packed cells in 15 ml culture medium. When cells were given into culture after performing an experiment, they were cultured in 6 well plates with 3 ml culture medium containing 50 µl of packed cells.

The state of the cultures and the parasitaemia were determined by daily Giemsa staining of thin blood films. When parasitaemia reached levels above 5 %, cultures were split to maintain constant culture conditions. For splitting, about 80 % of RBCs were discarded and an adequate volume of fresh RBCs was added to the culture.

For synchronization experiments, the culture medium within the flasks was changed and RBCs left for equilibration within new medium for at least 10 minutes. Then, the cells were taken from cultures and centrifuged 5 minutes at room temperature at 900 g. Haematocrit was adjusted to 10 %.

4.1.2 Purification and Synchronization of Schizonts

For this experiment, a 3 ml column was filled with 1000 mg of steel wool and autoclaved. The outlet of the column was connected to the 180° female Luer port of a two-way stopcock. Medium A was prepared, consisting of an aqueous phosphate buffered solution containing 0.5 % gelatine, 9.7 % sucrose and 1 mg/ml glucose. A syringe containing medium A was connected to the 180° male Luer port and the column was filled reversely with the solution. Air bubbles were evacuated by gentle finger tapping. After connecting the 180° male Luer port to a 20 G, 1 “ (equivalents 0.89 mm, 25.4 mm) needle, the column was positioned between the poles of the HGMS magnet. The magnet had a spacing of 1.3 cm. The system was equilibrated for 10 minutes at room temperature. To complete the

setting, a 50 ml syringe filled with 45 ml of medium A connected to an 18 G, 1.5 ” (equivalents 1.24 mm, 38.1 mm) needle by a two-way stopcock was placed on top of the opening of the column in the magnetic field. The setting is shown in Figure 9.

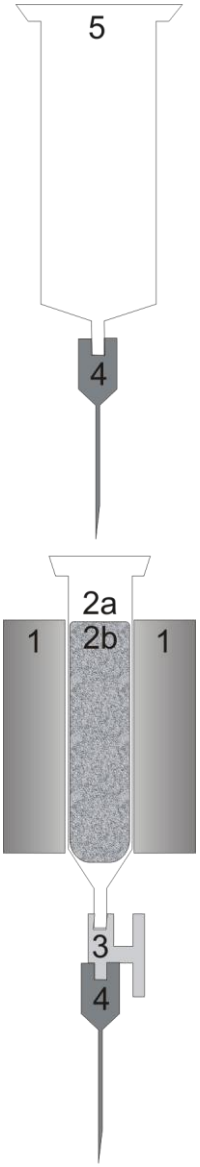

Figure 9

The high gradient magnetic separation column
(1) is the dipole magnet generating the high gradient magnetic field. Between the poles is the column (2 a) with the steel wool matrix (2 b) The column is connected to a two-way stopcock (3) and a 20 G needle (4). On top of the column is the 50 ml syringe containing medium A (5) connected to another needle (4).

For purification, 200 µl of packed cells were suspended in 20 ml of medium A. The outlet of the column was opened and the blood suspension slowly added on top of the column. We paid attention to never let the matrix run dry during the experiment. When the cell suspension had been applied completely, the outlet of the 50 ml syringe was opened and

the column rinsed with 45 ml of medium A. The flow velocity was constant over the time of the experiment as determined by the 20 G needle at the outlet of the 5 ml column. When washing was completed, the column outlet was closed. The column was taken out of the magnetic field to release paramagnetic cells from the matrix. A 10 ml syringe containing 12 ml of medium A was connected to the two-way stopcock and the column was flushed reversely. The eluate was collected and centrifuged 5 minutes at room temperature at 900 g. The pellet was resuspended in the culture medium described above (see 4.1.1) and an adequate volume of fresh RBCs was added.

4.1.3 Synchronization of Ring-Stage Parasites by Depletion

Two different carrier solutions were prepared for the experiments, medium B and medium C. Both were prepared from RPMI 1640 containing HEPES 5.94 g/l, glucose 1 g/l, hypoxanthine 50 mg/l, 5 % sodium bicarbonate. 0.2 % and 1 % of BSA were diluted to produce medium B and C, respectively. Both solutions were filtered through a 45 µm filter. For depletion, the 180° female Luer of a three-way stopcock was connected to a sterilized HGMS column consisting of a 5 ml column filled with 4000 mg of stainless steel wool. A 23 G, 1 “ (= 0.61 mm, 25.4 mm) injection needle was connected to the 180° male Luer port of the three-way stopcock as flow resistor. The column was positioned upright and filled reversely with medium C via the 90° female Luer port of the three-way stopcock. Air bubbles were evacuated by gentle finger tapping, the column positioned between the poles of the HGMS dipole magnet and equilibrated for 10 minutes at room temperature. The setting is virtually the same as in Figure 9. It only requires a three-way instead of a two-way stop-cock, a 23 G instead of a 20 G needle, and does not require the 50 ml syringe on top. The dipole magnet had a spacing of 1.4 cm. 50 µl packed cells suspended in 1500 µl of medium B were added on top of the column and the stopcock opened until the suspension had entered the matrix of the column completely. To optimize binding of paramagnetic cells to the matrix, the column was incubated for 30 minutes. Under these conditions, all paramagnetic cells bound to the steel wool. Then it was rinsed with 8 ml medium B and the flow-through was collected. The flow-through was centrifuged 5 minutes at room temperature at 900 g, washed once with 10 ml of RPMI, and the cells were re-suspended in culture medium and cultured as described above (see 4.1.1).

4.1.4 Synchronization by Sorbitol Lysis

The method of Lambros and Vanderberg (Lambros & Vanderberg, 1979) was applied. 50 µl packed cells where suspended in 1 ml of aqueous 5 % D-sorbitol solution and left in a water bath at 37 °C for 15 minutes. Then they were centrifuged at 900 g for 5 minutes and the supernatant discarded. The lysed late-stage infected erythrocytes on top of the packed cells, recognizable as a small darker film, were also discarded. Afterwards the cells were washed once in 10 ml RPMI and cultured as described above.

4.1.5 Staining of Blood Films for Enumeration of Parasitaemia and Morphological Analysis of Parasite Stages

Air dried blood films were fixed with methanol, stained with Giemsa stain for 5 minutes and afterwards analysed under the microscope.

For the analysis, 1000 cells or at least 500 parasitized cells were counted. The classification of stages was adapted (Lang & Löscher, 2000). Figure 10 shows examples of stages of development.

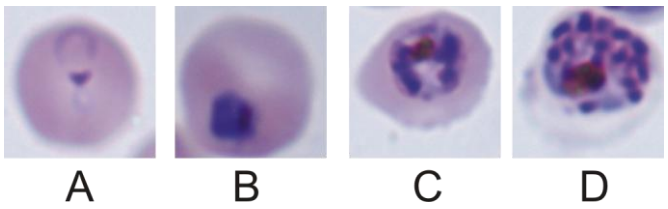

Figure 10

Parasites in different stages of development
The micrographs show parasites in different stages. The infected RBC carries an ring-stage parasite in (A), a trophozoite in (B), a schizont in (C), and a segmented schizont in (D).

4.1.6 Flow Cytometric Analysis of Cells Stained with AO

This staining procedure was adapted from Bhakdi et al. (Bhakdi et al., 2007). 1 µl of packed cells was suspended in 190 µl of PBS. 10 µl of AO stock solution (10 µg/ml in PBS) were added. The cells were incubated in the dark for at least 5 minutes before analysis by flow cytometry. This fast protocol allows distinction of non-infected from infected RBCs as well as an distribution of infected cells according to parasite maturation. This is due to increasing fluorescence with increase of RNA/DNA content.

4.1.7 Flow Cytometric Analysis of Cells Stained with HE

The method was adapted from Pattanapanyasat (Pattanapanyasat et al., 2003). 50 µl of cell culture with a haematocrit about 5 % were given into a flow tube and 5 µl of HE solution were added (1:10). The mixture was incubated in the dark at 37°C for 30 minutes. It could be kept in a refrigerator in the dark at around 8°C for several hours until acquisition. Before measurement, 500 µl of PBS were added to generate a suitable cell density for flow cytometric analysis. The procedure is more time consuming than AO staining, but it allows clear discrimination of three fractions: non-infected, ring-stage, and late-stage infected RBCs.

4.1.8 Analysis of Development of Cells in Culture after Experiments

HE staining was performed for the analysis of cell development in culture. The plotted histogram of HE was marked with the regions *M 1* for non-infected RBCs, *M 2* for ring-stage infected RBCs and *M 3* for late-stage infected RBCs. The fraction f_{ring} of ring-stage parasitized RBCs was calculated by

$$f_{ring} = \frac{n_{M2}}{n_{M2} + n_{M3}}.$$

n_{M2} is the number of cells counted within marker M 2 and n_{M3} those within marker M 3.
The calculation for the fraction f_{late} of late-stage parasitized RBCs out of all parasitized RBCs was

$$f_{late} = \frac{n_{M3}}{n_{M2} + n_{M3}}$$

An example is given in Figure 11, where histogram A shows a synchronized culture with mainly ring-stage infected RBCs, which have a fraction f_{ring} of 89.51 % out of all infected RBCs. In contrast, histogram B shows a synchronized culture with mainly late-stage infected RBCs, which have a fraction f_{late} of 85.71 %.

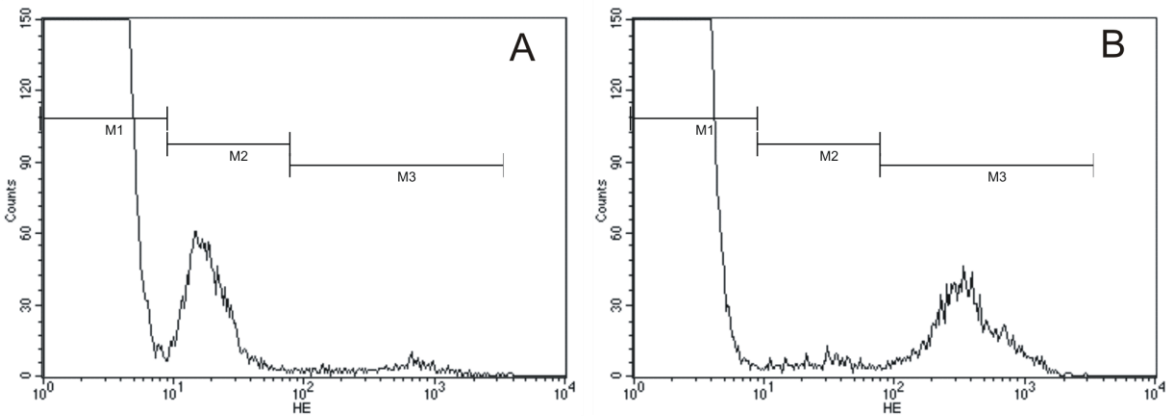

Figure 11

Flow cytometric analysis of synchronized cultures stained with HE

Flow cytometric histogram with events counted on the HE channel. (A) shows a synchronized culture where most infected cells are in ring-stage development, with M 1 representing 94.66 % non-infected RBCs, M 2 representing 4.78 % ring-stage infected RBCs and M 3 representing 0.56 % late-stage infected RBCs. (B) shows a synchronized culture where most parasitized cells are in late stages of development, with M 1 representing 94.61 %, M 2 representing 0.77 % and M 3 representing 4.62 % of the cells.

5 Results

5.1 HGMS for Isolation of Parasitized Cells

5.1.1 Methodology

We developed a method that allows purification of late-stage infected RBCs. The column is filled with medium and after an equilibration time, a RBC suspension is run through the column, followed by a washing procedure. The column is then removed from the magnetic field to flush off and collect the purified cells. There are a number of critical variables within this process.

First, we needed to check whether our experimental setting had any influence on the viability of parasites in the RBCs. As outlined in the Introduction, some authors fear a negative influence of magnetic fields or steel wool. To test this, a culture was left at room temperature under different conditions for 40 minutes. Standard samples were RBCs suspended in culture medium (1). In parallel, cells were suspended in medium together with immersed steel wool (2). A third sample was placed into the magnetic field (3). Thereafter, the development of the cultures was analysed for three days until high parasitaemia levels of around 30 % were reached. All cultures developed very similarly, indicating that our experimental setting had no negative influence on the parasites. Results of a representative experiment are given in Table 1 .

Day of culture development	Parasitaemia [%]		
	Standard (1)	Steel wool (2)	Magnetic field (3)
1	6.9	7.1	8.2
2	23.1	21.4	23.0
3	29.2	32.4	30.0

Table 1

The table shows the parasitaemia of different cultures over three days of culture development. The different samples are given in the columns, the day of development in the rows.

The development of a suitable carrier solution had a key role in the development of the procedure. The method required a solution that saturated unspecific binding sites, which would otherwise cause unspecific absorption of RBCs and impede purification. This problem was solved simply and effectively by adding gelatine to the solutions. This was

found to abrogate all non-specific absorptive interactions due to its negative charge. To avoid rouleaux formation of RBCs under the influence of gelatine, we raised the repulsive forces of the cells by replacing NaCl with the non-ionic disaccharide sucrose. Implementation of this sucrose-gelatine solution was a decisive step leading to success of the magnetic separation procedure.

A prerequisite for the separation process is the oxygenation of RBCs, which influences their magnetic properties. After oxygenation, only late-stage infected RBCs that contain haemozoin are paramagnetic and therefore captured within the column. Introduction of a 10 minute equilibration period wherein cell cultures were kept at room atmosphere was found to be sufficient for the necessary oxygenation of cells to take place. This step was almost trivial, yet of critical importance. It has not been heeded in the few recently published HGMS-based protocols for isolation of parasitized cells (Trang et al., 2004; Ribaut et al., 2008).

Flow velocity of the fluid through the column was another parameter that required careful attention. Velocity was varied by use of different needle sizes as flow regulators. High flow velocities led to good purification of late-stage infected cells, but with low yield. In contrast, low flow velocities led to high cell retrievement at the cost of low purity. The use of a needle of 20 G, 1“ was empirically found to provide ideal flow velocity and satisfactory purification.

To determine the capacity of the purification column, we counted the number of isolated cells in a Neubauer chamber. The suspension contained up to 17.4 $x10^6$ RBCs, which equalled 1.7 µl of packed cells. The mean number was 9.89 $x10^6$ RBCs, ± 3.89 $x10^6$ (S.D., n=7). The mean number of captured cells equalled around 1 µl of parasitized RBCs. This was enough to start cultures in four 25 cm² culture flasks. The infected cultures had a parasitaemia of 1 % after a few hours when cells had undergone cell rupture and the parasites had reinvaded RBCs.

The recovery rate describes the percentage of retrieved cells out of total late-stage infected RBCs that were applied to the column. The recovery rate was 4 %, ±1.61 % (S.D., n=7). The number of parasitized RBCs applied to the column influenced the purification results. The fraction of schizont infected cells within the isolated population increased when more cells were applied. This represents a quality increase towards a smaller age distribution. Variation in the number of cells applied to the column had no impact on the purity. Purification rates were consistently high. Table 2 shows the analyses of two representative experiments. Cells for both experiments were taken from the same culture. The

haematocrit of the cell suspension was kept at 1 %. In effect, 100 µl of packed cells were suspended in 10 ml medium or 300 µl in 30 ml. A highly purified population of infected RBCs was obtained in both experiments. Yet, the experiment with 100 µl of packed cells let to recovery of a relatively wide age distribution of parasite development: 46.4 % of the purified cells contained trophozoites and only 51.6 % contained schizonts. The fraction of schizont parasitized cells increased to 82.2 % in the other experiment. For the standard protocol, column loading was empirically fixed to 200 µl of packed cells. This yielded high fractions of schizont parasitized cells and allowed optimized cell culture continuation. We further used cultures with a high parasitaemia of around 20 % to assure high numbers of late-stage infected cells.

RBCs [µl]	Purification [%]	Non infected RBCs [%]	Trophozoite infected RBCs [%]	Schizont infected RBCs [%]
100	98.0	2.0	46.4	51.6
300	98.4	1.6	16.2	82.2

Table 2

The table shows purification results of two representative experiments, acquired by flow cytometric analysis. The columns show the total purification of infected RBCs, the fraction of non-, trophozoite- and schizont-infected RBCs. The different experiments are listed in the rows.

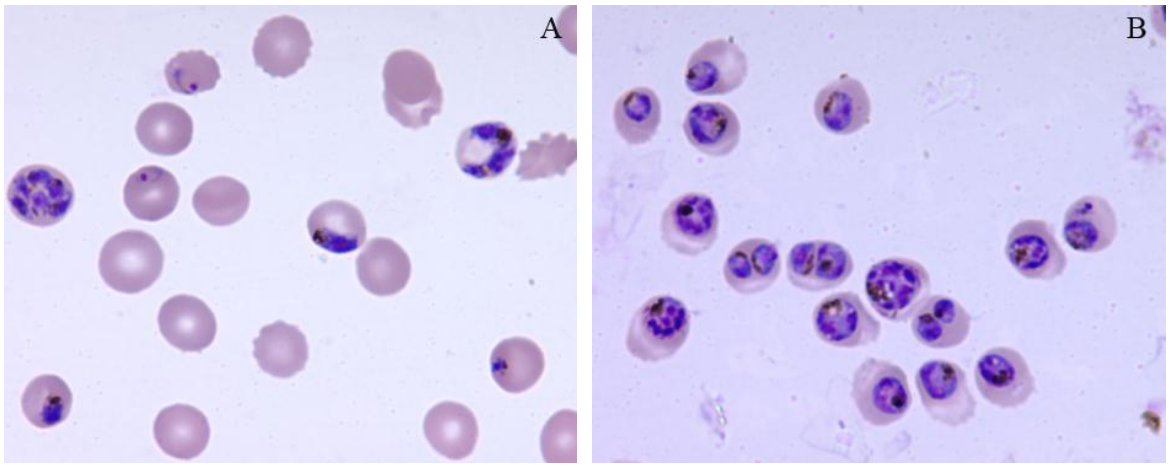

Figure 12

Micrographs of blood films
(A) shows the starting culture. It had a parasitaemia of 21.27 % (B) shows the experimental result. After purification, the slide contains mostly late-stage infected RBCs. The infected cells have been purified to 95.4 %.

5.1.2 Results

The protocol permits isolation of late-stage infected RBCs to purification levels of 99 %. The mean purification rate in ten experiments was 96.84 % ± 1.78 % (S.D., n=10). This underscores the high reliability of the method. Analysis was done by blood smears or AO staining.

A representative result is shown in Figure 12. On the left and right are blood films of the culture before and after the purification procedure, respectively. The culture had a high parasitaemia of 21.27 % and hence many late-stage infected RBCs. Virtually all cells eluted from the magnetic separation column were parasitized and harboured schizonts.

Flow cytometric analyses of the cells stained with Acridine orange (AO) were performed in order to obtain sound quantitative data. This procedure was recently optimized for the detection of parasitized RBCs in this laboratory (Bhakdi et al., 2007). AO stained cells show increasing fluorescence with increase of RNA/DNA content. This allows distinction of non-infected from infected RBCs as well as an distribution of infected cells according to parasite maturation. However, especially for late-stage parasitized RBCs, stage populations merge and cannot be clearly distinguished. Figure 13 shows histograms and dot plots of cells before and after magnetic column purification in another representative experiment. The histogram of the starting culture (Figure 13 A) shows a peak in the marker region M 1, which represents non-infected RBCs. This peak does not appear in the histogram taken after the purification (Figure 13 C). The dot plots show corresponding results (Figure 13 B, D). The region of non-infected RBCs is marked. In the starting culture, 86.49 % are within

this region. After purification, only 0.44 % non-infected RBCs were counted within this region and infected cells comprised over 99 % of the total cell population.

Blood films allowed stage specific analysis after purification. The isolated infected cells consisted of trophozoites and schizonts only. There were no ring stage infected erythrocytes any more. The fraction of a certain stage was calculated as percentage of total infected cells. The schizont fraction approached 92.0 % with an average of 78.31 % and a standard deviation of 14.13 %. These numbers were calculated on the basis of eight experiments. The fraction of schizonts varied. As described above, one factor influencing the final distribution of infected cells is the number of cells applied to the column.

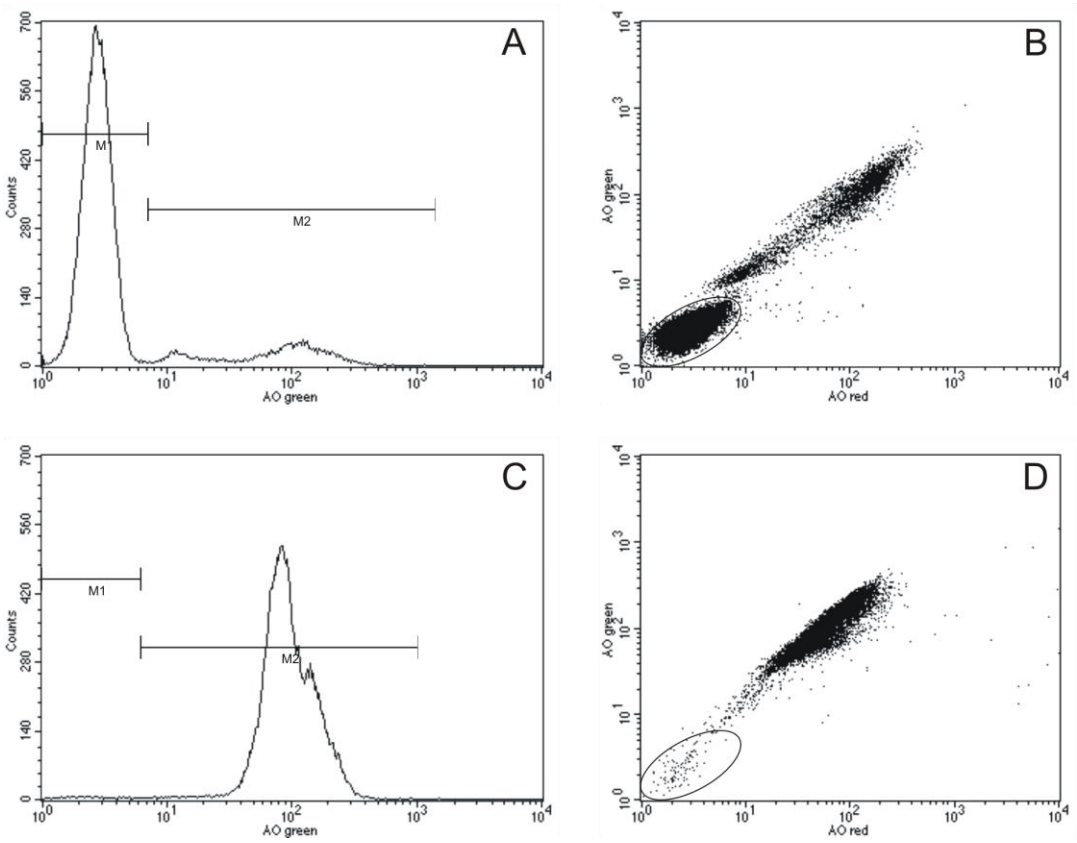

Figure 13

Flow cytometric analysis of cultures stained with AO

Starting culture and cells after purification in a representative experiment. The starting culture has a parasitaemia of 13.51 %. After purification 99.54 % are infected RBCs in late stages of development. In the histograms M 1 represents non-infected RBCs and M 2 represents infected RBCs. The marked region in the dot plots marks non-infected RBCs. (A) shows the histogram and (B) the dot plot of the culture taken for the experiment. (C) and (D) show analysis after the procedure. The absolute fluorescence of the counts in both samples cannot be directly compared. The in total diminished fluorescence of late-stage infected cells in (C) and (D) is an artefact. This has been confirmed by parallel analysis of blood films. It might be due to traces of sucrose in the culture after magnetic purification

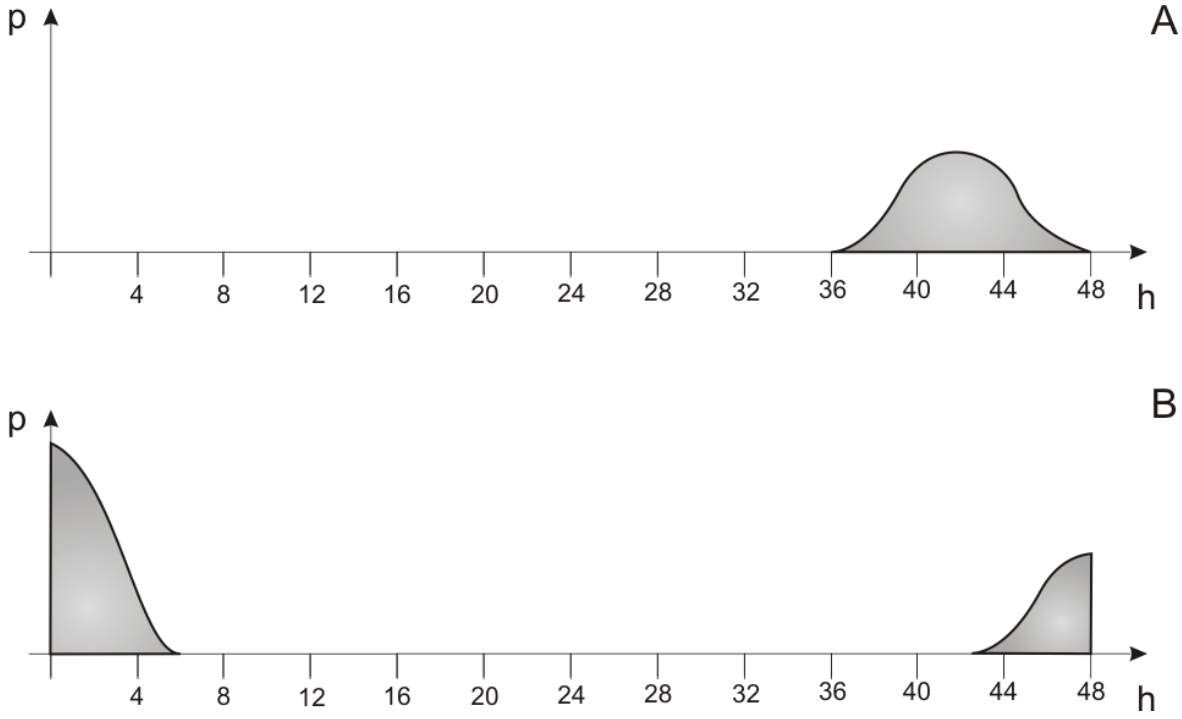

Figure 14

Schematic drawing of the principle of "fine tuning"
On the x-axis the age of parasites within infected RBCs is plotted in hours (h). On the y-axis is the number of parasites (p) in arbitrary units. (A) shows a pre-synchronized culture. The parasites within infected RBCs have an age distribution from 36 h to 48 h. In (B), the same culture is shown 6 hours later. After cell rupture and reinvasion, the number of parasites has increased. Those parasites are not magnetically susceptible and can be seen in the population from 0 h to 6 h of development. The remaining susceptible parasites have a reduced age distribution of 42 h to 48 h.

"Fine-tuning" by repeated column separation

Vanderberg described "fine tuning" of the sorbitol lysis for a more tightly synchronized cell culture. He demonstrated the utility of repeating the lysis procedure after a certain time interval to further decline the age distribution of the synchronized cells. Inspired by this, we developed fine tuning for our purification protocol, the principle of which is schematically illustrated in Figure 14.

Parasitized RBCs in a synchronized culture have a certain age distribution. When the population reaches the schizont stage, older infected RBCs rupture and merozoites invade other RBCs. In that very moment, they lose their magnetic susceptibility and will no longer be caught in the purification column. The remaining cells have a correspondingly smaller age distribution. A repeat run through the magnetic column leads to their isolation.

The fine-tuning protocol for our separation procedure capitalized on this and was as follows. The first magnetic column separation was undertaken as described above after 2-3 days of primary culture. The parasitized cells obtained were then re-cultured for 4 days, and the column run was repeated. Figure 15 depicts the results of an experiment. Fine-

tuning led to isolation of cells all harbouring schizonts, the majority of which were already segmented.

To sum, the HGMS procedure is the first method that made it possible to isolate late-stage parasitized cells in a single separation step from cell cultures.

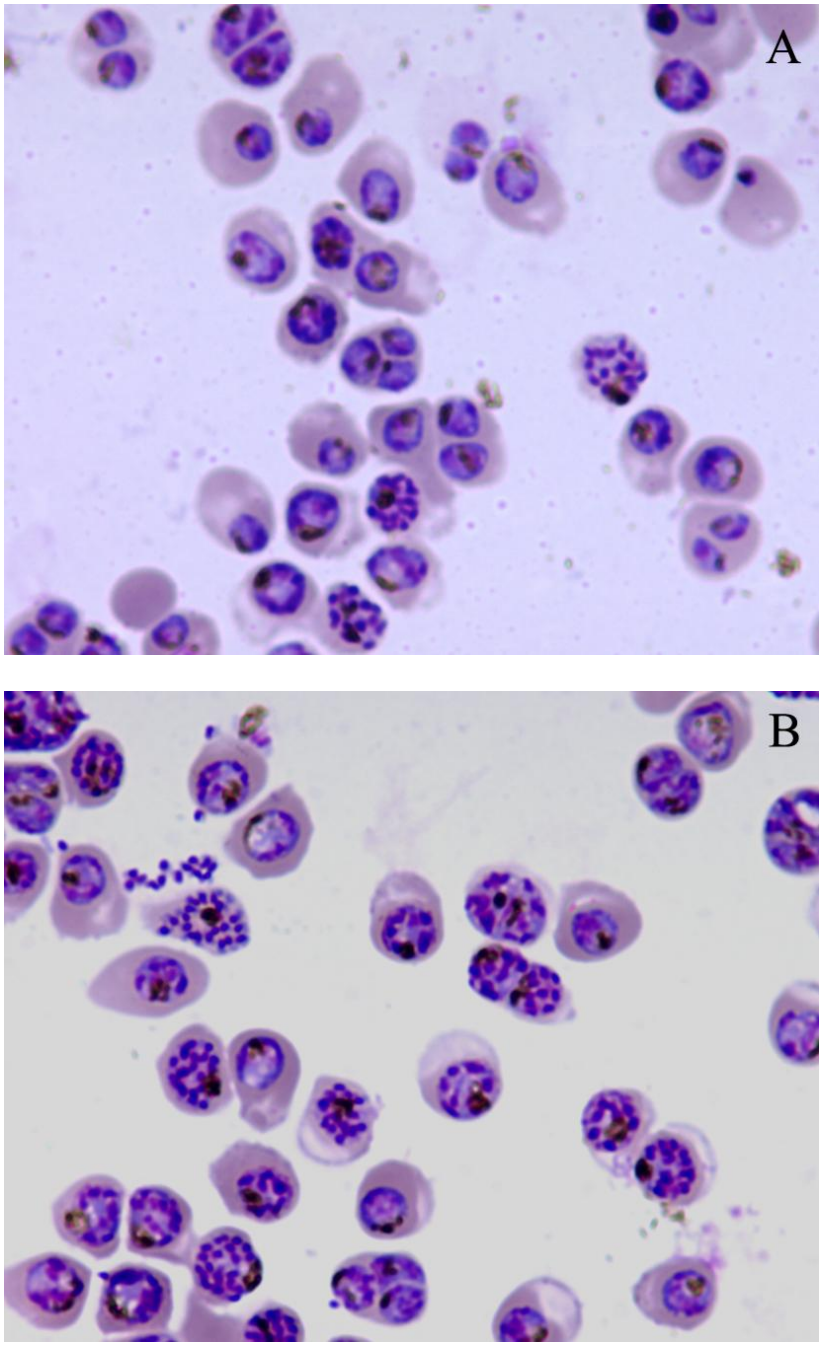

Figure 15

Micrographs of blood films
(A) Specimen obtained after a standard purification. (B) Specimen obtained after "fine-tuning" four days after the first isolation procedure, showing the presence of more schizont parasitized cells and only few trophozoite parasitized cells. Quite a number of schizonts have segmented already.

5.1.3 Synchronization

We wanted to check which level of synchronization is reached after performance of the purification protocol. At present, sorbitol lysis is generally considered the standard procedure for synchronization. We therefore compared culture development after HGMS purification with culture development after sorbitol lysis. Our isolation protocol achieves purification of late-stage infected RBCs while sorbitol lysis commences synchronization at ring-stage level, so comparison could not be started on the same day of culture development. We started comparison on day 1 of culture after sorbitol lysis with day 2 of culture of cells retrieved from the HGMS columns, when parasites had developed into late stages. For the sake of clarity this is set as day 1 for both cultures in the following.

Purified cells mixed with non-infected RBCs, or cells synchronized by sorbitol lysis were given into culture and flow cytometric analysis was performed once daily. Cells were stained with Hydroethidine (HE) before analysis. HE staining was preferred to AO staining, because it allows clear discrimination of three fractions: non-infected, ring-stage, and late-stage infected RBCs. Figure 16 shows a representative example of a culture that is synchronized in late stages of development. Both methods were performed three times and the mean was compared.

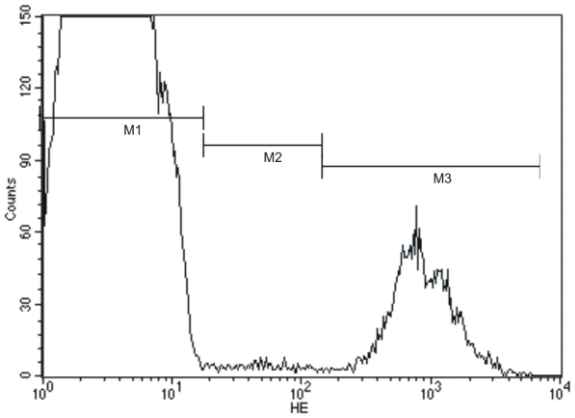

Figure 16

Flow cytometric analysis of a synchronized culture stained with HE

Flow cytometric histogram with events counted on the HE channel. The histogram shows a synchronized culture where most infected cells are in late stages of development, with M 1 representing 93.11 % non-infected RBCs, M 2 representing 0.48 % ring-stage infected RBCs and M 3 representing 6.41 % late-stage infected RBCs. 93.03 % of total infected RBCs are in late stages of development.

The data are shown in Figure 17 and the values are given in Table 3. Cultures developed similarly after performance of both methods. Synchronicity gradually decreased from day to day. This was as expected, since the step of synchronization sets all infected cells to the same developmental stage, but does not prevent the parasites to develop at different speeds under culture conditions.

These results show that synchronization by the purification protocol is as good as sorbitol lysis. Once more, it underlines the fact that viability of the parasites is not affected by HGMS separation.

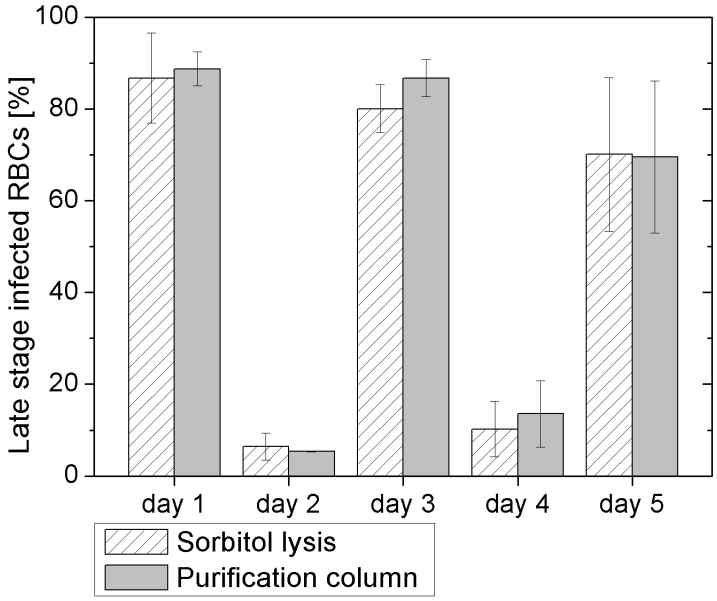

Figure 17

Day of culture development	Ring-stage fraction [%]		Late-stage fraction [%]	
	Sorbitol lysis	Purification column	Sorbitol lysis	Purification column
1	13.26	11.27	86.74	88.73
2	93.57	94.65	6.43	5.35
3	19.96	13.26	80.04	86.74
4	89.77	86.45	10.23	13.55
5	29.93	30.44	70.07	69.56

Table 3

Culture development after sorbitol lysis and HGMS based cell isolation

In Figure 17 and Table 3 the respective fractions are given in percent of total infected cells. The columns represent the mean number and the error bars the standard deviation that was calculated from three data sets. Striped columns show culture development after sorbitol lysis and grey columns development after magnetic purification. The second day of culture development after HGMS of late-stage fraction was set as day 1, to be able to compare it to sorbitol lysis. The error bar on day 2 after purification is to small to be depicted.

5.2 HGMS for Removal of Parasitized Cells from Cultures

5.2.1 Methodology

In complementation to the purification procedure, we developed a protocol that allows removal of all paramagnetic, late-stage infected RBCs from a cell suspension. The procedure is very similar to the purification protocol, except for a 30 minute incubation time for the cells within the column to optimize binding of all paramagnetic cells. Furthermore the column was washed within the magnetic field. This kept the late-stage infected RBCs bound to the matrix of the column and depleted cells could be collected.

Again, a suitable carrier solution was of central importance for the procedure. Since cells remain in the carrier solution for up to one hour, we designed medium B, which is very similar to culture medium. The expensive human AB serum was replaced by 0.2 % of BSA. To avoid sedimentation of RBCs during the incubation time, the column had to be filled with a denser medium. Therefore we designed medium C that contained 1 % BSA.

This protocol was optimized for the capture of all late-stage infected, magnetically susceptible cells. Some non-infected and ring-stage infected cells also adhered to the column due to unspecific absorption. It was important to stay within the capacity limits of the column. The experiment was performed successfully up to a late-stage parasitaemia of 4.8 % which equals cell capture of 24 million late-stage parasitized RBCs for the total number of $5x10^8$ RBCs run through the column. The loading capacity was equivalent to two 25 cm^2 culture flasks of synchronized cells with a parasitaemia of 1 %.

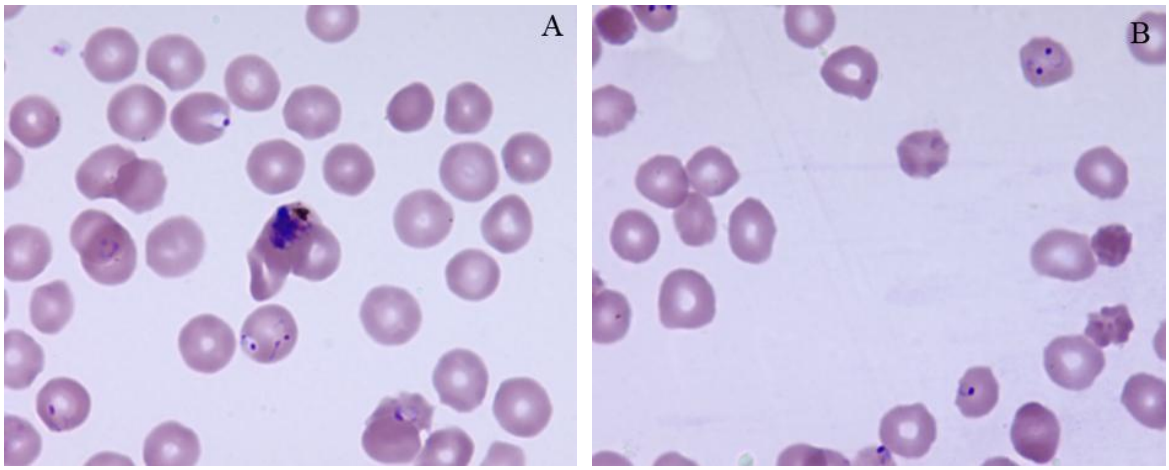

Figure 18

Micrographs of blood films
(A) shows a blood films of an infected blood culture with 9.7 % parasitaemia. (B) shows a blood film after magnetic depletion. The depleted cell population had 6.3 % parasitaemia and 97.1 % of the parasitized cells were in ring-stage.

5.2.2 Results

The method permits removal of late-stage parasitized RBCs from an infected blood culture. Virtually all parasitized cells remaining in a blood culture after depletion are in ring-stage development (98.27 %, ± 1.31 % (S.D., n= 7)).

A representative result is shown in Figure 18. On the left and right are blood films of the culture before and after the magnetic depletion, respectively. The culture had a parasitaemia of 9.7 % and showed infected cells in different stages of development. After magnetic depletion, virtually all parasitized RBCs were in ring-stage development. Further the parasitaemia decreased to 6.3 % which underlined the removal of cells.

For sound quantitative data, flow cytometric analysis was performed with AO stained cells. Besides distinction of non-infected from infected RBCs, AO staining yields increasing fluorescence with the increase of RNA/DNA content. This is correlated to parasite age. A representative result is shown in Figure 19. Histogram and dot plot of the culture before (A, B), and after magnetic depletion (C, D), and after sorbitol lysis (E, F) are shown. There are hardly any counts in the histogram after depletion with fluorescence intensity higher than 40 in the channel of AO green. Before the experiment the blood culture had counts up to a fluorescence intensity of 200. Similarly, in the dot plots the depleted cell population showed hardly any dots in the marked region of late-stage infected cells. Cells with high fluorescence had been successfully removed from the culture. Removal of cells was

underlined by parasitaemia levels. The culture taken for the experiment had a parasitaemia of 6.72 % which decreased to 4.28 % after depletion.

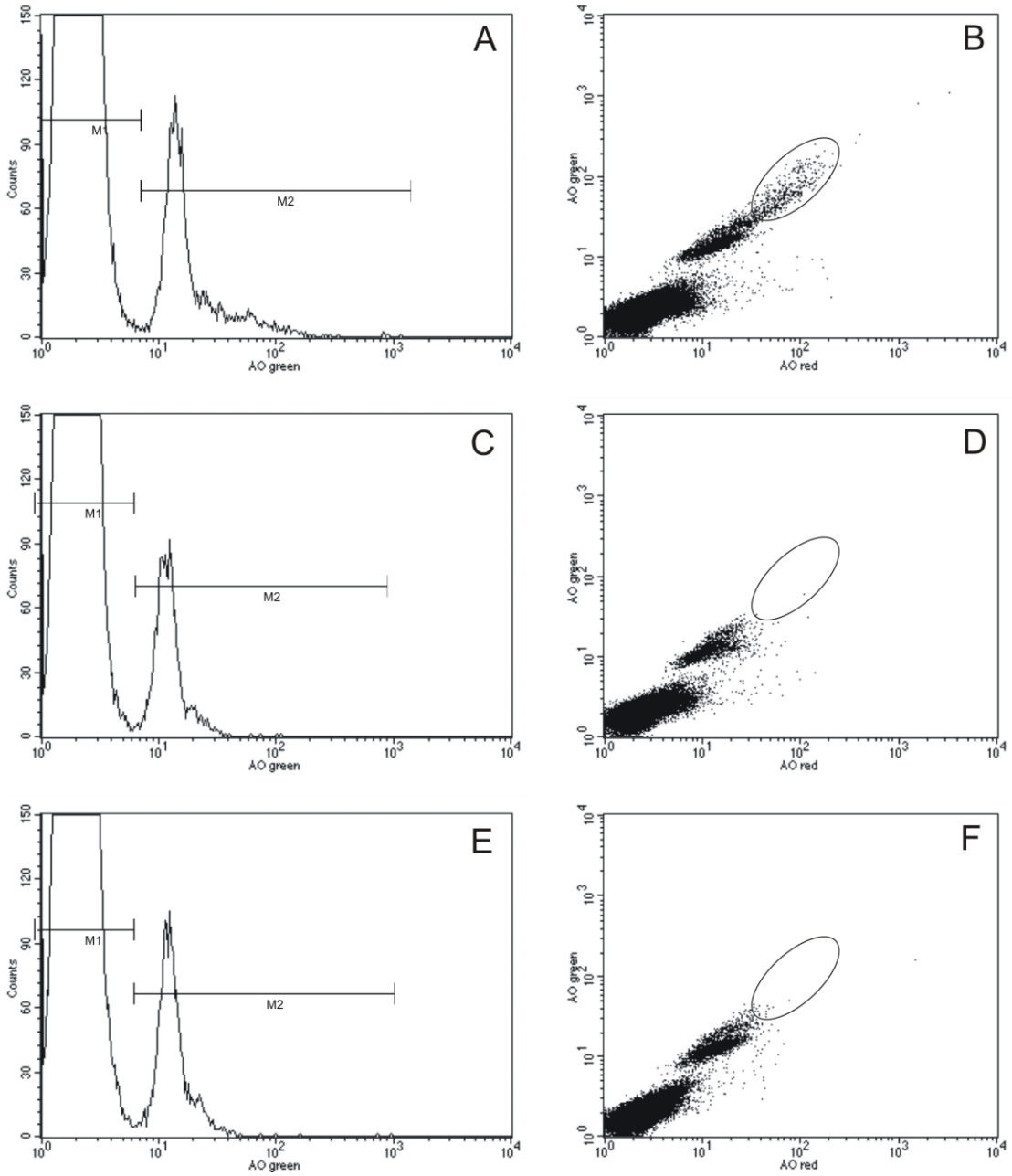

Figure 19

Flow cytometric analysis of cultures stained with AO
(A) and (B) show histogram and dot plot of the cell culture taken for the experiment, (C) and (D) show analysis after magnetic depletion and (E) and (F) analysis after sorbitol lysis. In the histograms, M 1 represents non-infected RBCs and M 2 infected ones. In the dot plots the marked region shows the counts for late-stage infected RBCs.

5.2.3 Synchronization

Again, we wanted to check the levels of synchronization attainable by the new procedure and therefore compared culture development after magnetic depletion to that after sorbitol lysis. Both methods result in a cell population synchronized in ring-stage development. Therefore direct comparison was possible.

Flow cytometric analysis with HE stained RBCs was performed once daily. Figure 20 gives a representative example of a culture synchronized in ring stages of development. The comparison was done with the mean out of three experiments for each procedure.

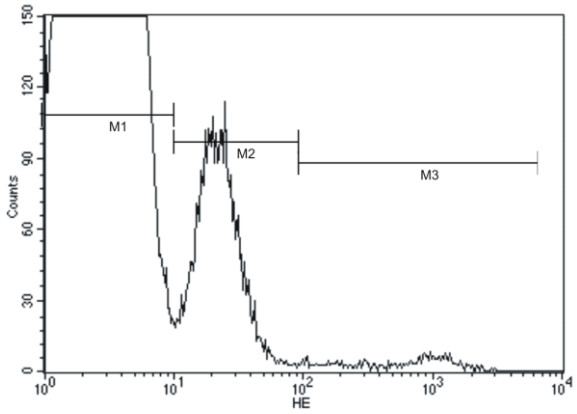

Figure 20

Flow cytometric analysis of a synchronized culture stained with HE
The histogram shows a synchronized culture where most infected cells are in ring-stage, with M 1 comprising 90.28 %, M 2 comprising 9.08 % and M 3 comprising 0.64 % of total cells. 93.41 % of total infected cells are in ring stages of development.

A direct comparison of the two methods is shown in Figure 21 and the values are given in Table 4. Both methods result in a similar extent of synchronization and both gradually desynchronize in a comparable way.

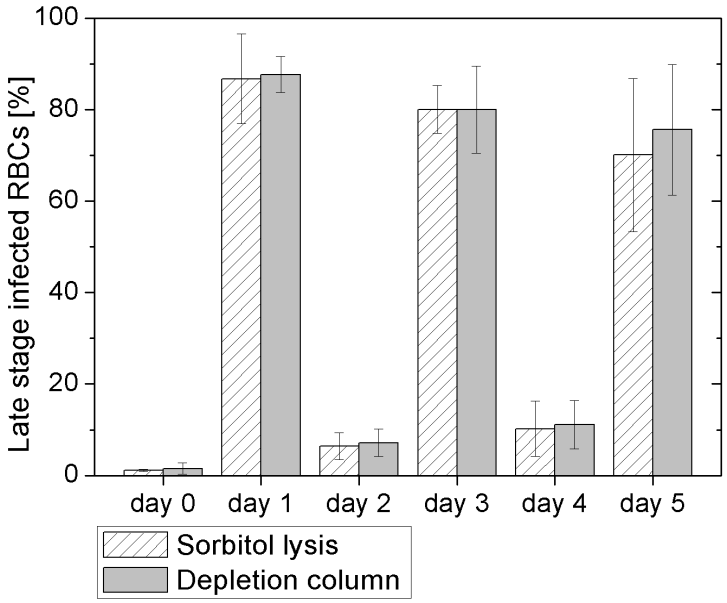

Figure 21

Day of culture development	Ring-stage fraction [%]		Late-stage fraction [%]	
	Sorbitol lysis	Depletion column	Sorbitol lysis	Depletion column
0	98.86	98.48	1.14	1.52
1	13.26	12.33	86.74	87.67
2	93.57	92.84	6.43	7.16
3	19.96	19.97	80.04	80.03
4	89.77	88.87	10.23	11.13
5	29.93	24.40	70.07	75.60

Table 4

Culture development after sorbitol lysis and magnetic depletion
In the figure, late-stage infected RBCs are depicted in % of all infected RBCs. The columns represent the mean number and the error bars the standard deviation that was calculated from three data sets. Striped columns show culture development after sorbitol lysis and grey columns development after magnetic depletion. The table gives the values for both, ring-stage and late-stage fraction and refers to the figure on top.

6 Discussion

6.1 Contextualisation of the Results

The presented work is a comprehensive approach to apply high gradient magnetic separation (HGMS) to RBCs infected with *Plasmodium falciparum.* The purification protocol allows purification rates of nearly one hundred percent. This is of distinct interest. As will be outlined in the following, no alternative method exists for obtaining such pure preparations of late-stage parasitized cells infected with *Plasmodium falciparum* with similar ease, reproducibility and cost-effectiveness.

To date there are two standard procedures for the purification of late-stage infected RBCs: The first is the fractionation with Percoll gradients and the second method is gelatine flotation. Percoll gradient centrifugation has reported purification rates in the range of 76 to 98 % (Nillni, Londner & Spira, 1981; Kramer, Kan & Siddiqui, 1982; Rivadeneira, Wassermann & Espinal, 1983; Dluzewski et al., 1984; Trang et al., 2004). The purification rates reached by our protocol are comparable to the maximum rates reported for Percoll gradients. The new protocol strongly prevails over Percoll centrifugation in the consistency of the results. Percoll carries an intrinsic limitation, because there are always some non-infected RBCs that show the low density otherwise characteristic of late-stage infected cells. The number of low density uninfected RBCs increases with storage time (Willimann et al., 1997). This is disadvantageous, because it necessitates frequent RBC preparation from fresh blood. Our newly introduced protocol has no such intrinsic limitation and purification rates up to 99.56 % were attained in a single column run.

Even with little handling experience, the newly developed procedure can be completed in less than 45 minutes - including washing and oxygenation of malaria cultures as preparation for the experiment. Compared to separation by Percoll gradients, the new protocol is much more time efficient.

When further fractionation of infected cells is needed, separation can be carried out with discontinuous Percoll-sorbitol gradients (Kutner et al., 1985; Deharo et al., 1994; Krugliak & Ginsburg, 2006). Yet, this is an even more difficult and time consuming procedure that requires several washing steps after cell selection. Further fractionation is promoted by sorbitol influx into parasitized RBCs. A possible negative impact of sorbitol within the cells cannot be excluded. We observed RBCs to be unaffected in their viability after exposure to our buffer. There was no negative impact of sucrose. This was as expected,

since Krugliak et al. already showed that sucrose does not enter the new permeability pathways of infected RBCs (Ginsburg et al., 1983).

Costs of purification with Percoll gradients are comparatively low with 1.5 to 3 € per experiment for a conventional Percoll protocol requiring approximately 1.5 ml of Percoll solution per 200 µl packed cells plus culture medium for washing steps. The costs of the HGMS column and system buffers per experiment amounts to less than 5 €. These slightly higher costs are well compensated for by the major advantages the method offers.

The other standard procedure for late-stage purification is gelatine flotation. It has reported purification rates from 12 to 95 % (Jensen, 1978; Ansorge et al., 1996; Lelievre, Berry & Benoit-Vical, 2005), clearly inferior to the HGMS procedure. This method is furthermore limited to knob positive strains. Our procedure was developed with the strain TM 267 which is knob negative and can be utilized for any other *Plasmodium falciparum* strain.

During the last years, several publications reported the use of commercially available polymer coated columns for purification (Trang et al., 2004; Ribaut et al., 2008). The columns overcome the problem of unspecific absorption by using a hydrophilic polymer-coated matrix. With these columns, purities up to 99 % become possible. However, this approach is associated with significant costs.

We developed an entirely different approach by optimizing the buffer used in the system. The carrier fluid employed in our columns contains macromolecules that are capable of saturating unspecific binding sites. Thereby, polymer coating becomes unnecessary. Material costs of the present method are consequently very low.

A step in our procedure that deserves emphasis is the oxygenation of the RBCs. This parameter has been overlooked in the past. According to the theory regarding magnetic properties of haemoglobin, higher levels of purification are expected when all haemoglobin in the cells is oxygenated and diamagnetic, so that paramagnetic property is solely determined by haemozoin. The almost trivial step of deoxygenation was indeed of critical importance in guaranteeing the high reproducibility of our HGMS procedure.

After this general comparison, a closer scrutiny of the publications on the application of HGMS in malaria research is warranted. Trang et al. (Trang et al., 2004) described a method for one-step concentration of *Plasmodium berghei* from whole mouse blood, obtained by venous puncture. The experimental results indicated good purification (up to 98 %) of late-stage infected RBCs, but purification was measured in parasitaemia levels, which is not stage-specific analysis. Only the numbers of representative experiments were given, which does not give evidence about the consistency of the results. In contrast, we

have given stage-specific analysis that showed a high fraction of schizonts and has proven the high consistency of our results. Furthermore, we showed successful purification of 20 times more cells within one column. We expect our method to be suitable for the purification of *Plasmodium berghei* from whole blood as well.

The Valentin group described purification of infected RBCs for different species of *Plasmodium* (Ribaut et al., 2008). Again purification was measured in parasitaemia levels, which is not stage-specific and only the numbers derived from representative experiments were given, which does not testify consistency. Importantly, *Plasmodium falciparum* cultures were employed that were first synchronized by sorbitol lysis every 48 hours. This implies that their procedure could only be performed in a certain time window every other day when the culture was in late stages of development and contained paramagnetic cells. In contrast, our method appeals through the utter simplicity of handling. It can be performed at any time of culture development and unfailingly enables parasitized cells to be retrieved without significant contamination with non-infected or early-stage infected cells. This novelty marks an advance in the field. The Valentin group further described other applications for their method: purification of the haemozoin containing sexual stages of *Plasmodium falciparum* and purification of other *Plasmodium* species from peripheral blood of infected patients. All these are possible future applications of the present method.

There has been a customer report on the possibility of using HGMS to isolate *Plasmodium falciparum* parasitized RBCs. Purities of 70 to 90 % were given. Purities above 90 % were only achievable by using a second column. Selective concentration of schizont infected RBCs was not reported and data for maximum column capacity was not given (Uhlemann et al., 2000). The superiority of our HGMS procedure is apparent.

Taken together, the present method combines high reproducibility, simplicity and cost-effectiveness and may become of use to investigators engaged in many different areas of malaria research.

The possibilities of using HGMS as the starting point for culture synchronization have not been explored by other laboratories and we accordingly undertook this task. Culture synchronization is an application for which competing purification methods are used (Ginsburg et al., 1983; Hoppe, Verschoor & Louw, 1991). Synchronization of cultures following HGMS based cell isolation matched synchronization by the classic sorbitol lysis procedure. Many advantages exist over other methods that begin cultures with isolated, late-stage parasitized cells. Ease of handling compared to Percoll centrifugation and

superior purification rates compared to gelatine flotation are two that have been discussed above.

Further to its utility as a means to isolate late-stage parasitized cells, we explored the possibilities of employing the technique as a depletion procedure. A protocol was established that allowed essentially total removal of late-stage parasitized cells. This is the first report showing that HGMS can thus be used for selective retrieval of early-stage infected cells. Following HGMS depletion, highly synchronized cultures could again be obtained with high consistency matching the sorbitol lysis procedure.

As a positive trade-off, osmotic stress of the cells by exposure to sorbitol is avoided. The cells are within a medium which has a high similarity to the culture medium during the whole procedure. Our protocol is entirely reliable and the results are consistent.

To sum, two protocols are described that can both be used for establishing synchronous cultures. Development of these cultures was comparable to that observed after sorbitol lysis. This underlined the intrinsic characteristic of the parasites to develop at different speeds, which thereby gradually led to desynchronization of the cultures. As a consequence, it was never possible to obtain late-stage infected cells at a purity exceeding 90 % by synchronous culture.

6.2 Perspectives

Many advances in malaria research rest on the possibilities of studying parasites at specific stages of their development. Prerequisites for this are the availability of simple and cost-effective procedures to isolate parasitized cells and to maintain them in synchronized cultures. The present HGMS method expands the narrow spectrum of technologies that are currently available for such studies. Current magnetic separation techniques employ columns with a polymer-coated matrix. Our method exploits the use of an optimized buffer system, which renders polymer-coating unnecessary and dramatically reduces costs. The isolation procedure can accordingly be performed in laboratories with limited resources and equipment. The only instruments required are a centrifuge, a microscope, and a strong permanent magnet. Despite its simplicity, rapidity and cost-effectiveness, the method renders it possible to obtain late-stage parasitized cells in a state of purity and homogeneity that has not been attained by any other method hithertofore.

Perspectives for Clinical Diagnostics

One approach towards development of a screening test for malaria is based on semi-automatic flow cytometry, which might be performed with blood samples of febrile patients. Malaria pigment is present in white blood cells (WBCs), and it has been shown that pigment loading of neutrophils and monocytes correlates with severity of the disease (Lyke et al., 2003). It has also been observed that semi-immune individuals have higher levels of pigment containing monocytes than do individuals with a naive immune system. This fact is of high interest for people in endemic regions who are semi-immune. The described test has high specificity but lacks sensitivity. However, the authors showed that detection increased with higher concentrations of pigment carrying cells (Grobusch et al., 2003; Hänscheid, Egan & Grobusch, 2007). It should be possible to directly isolate such cells from blood samples with the present HGMS technique, and then simply count them with a conventional cytometer. Early diagnosis remains one fundamental factor influencing overall survival rates, so the successful introduction of such a procedure could directly impact on clinical medicine.

Isolation of pigment-containing white blood cells could be of special importance in pregnant women. Particularly in the case of *Plasmodium falciparum*, many infected RBCs sequester within the placenta, making diagnosis in blood films from peripheral blood difficult. However, pregnant women have pigment loaded WBCs in the peripheral blood

(Hänscheid, Egan & Grobusch, 2007), so that HGMS might offer a novel possibility to develop a highly sensitive and specific test for malaria diagnosis in these patients.

Perspectives for Basic Research

The HGMS technique could become a valuable aid to many groups engaged in the investigation of diverse aspects of growth, physiology and cell biology of *Plasmodium falciparum*. Through appropriate adjustments of the protocols, columns can be run in parallel to deplete cell cultures of later stage parasitized cell, or for selective isolation of the latter. Cultures containing quite uniform populations of either ring-stage infected cells or schizonts can thus be obtained from the same culture within less than one hour and without exposing the cells to sorbitol or centrifugation in density gradients. The potential usefulness, e.g. for the study of such complicated processes as protein trafficking between the parasite and host cell membrane (Lingelbach & Przyborski, 2006) should be obvious.

Studies on parasite physiology and metabolism should also be facilitated. A case in point is the analysis of haemozoin production. It is generally assumed that production of this metabolite is a linear process beginning from the time point of invasion (Moore et al., 2006). However, our results indicate that this assumption might require modification. Thus, HGMS-based capture of infected cells was only possible from a certain stage of parasite maturation onwards. Ring-stage infected erythrocytes showed no susceptibility whatsoever. It follows that haemozoin production may actually not occur to a relevant extent in the ring-stage of parasite development. Some interesting publications on the mechanisms of drug action would actually be inline with this contention. The cytotoxic mechanism of quinoline works via inhibition of haemozoin production. Intrinsic quinoline resistance has been described for the early ring-stage and late schizonts (Smalley, 1977; Slater, 1993; Sullivan, 2002), which would be in accord with our assumption that haemozoin production does not occur in the early stage of parasite development.

The HGMS procedure could be useful for isolation of gametocytes. Current production of these cells *in vitro* follows a complicated protocol lasting several days (Fivelman et al., 2007). The Baker group has introduced a new protocol which is the modification of a common protocol and replaced Percoll gradient centrifugation by HGMS purification. This led to higher degrees of purity. Our introduced isolation procedure should be suitable as well for this purification step in the protocol. The Baker group does not describe an incubation time for oxygenation of RBCs and again the argument of cost-effectiveness applies. We anticipate that our isolation protocol will also mark an advance in this area.

There is also high interest in obtaining homogenous populations of merozoites to study the process of invasion. One possibility is to isolate late-stage schizonts (Etzion, Murray & Perkins, 1991; Khattab et al., 2008), from which merozoites are released (Elford, 1993). Schizonts isolated by our protocol yield optimal starting samples for these experiments and should therefore provide researchers with an improved tool to isolate merozoites in the future.

Perspectives for Drug Sensitivity Testing

Surveillance of drug resistance in endemic regions and drug development is essential (Noedl, Wongsrichanalai & Wernsdorfer, 2003). New drugs are urgently needed, since parasite resistance is growing (Greenwood & Mutabingwa, 2002). When ring-stage and late-stage synchronized cells are to be studied as targets, both the presented HGMS protocols can be employed instead of sorbitol lysis and gelatine flotation, respectively (Druilhe et al., 2001).

HGMS for Purification and Depletion of Other Cells

The modified HGMS protocols should be applicable to many other separation processes for cells that show intrinsic magnetic susceptibility. They should be applicable for labeling and separating cells with monoclonal antibodies conjugated to nanosize magnetic particles. Cell separation technology has experienced major advances in the past decades and magnetic separation technologies have been established (Stewart et al., 2000). Pilot experiments performed in this laboratory already indicate that the improved buffer systems allow superior separation results to be achieved.

7 Synopsis

This thesis was undertaken to explore possible applications of high gradient magnetic separation (HGMS) for the separation of RBCs infected with *Plasmodium falciparum*, with the dual aim of establishing a novel and superior method for isolating late-stage infected cells, and of obtaining synchronized cell cultures.

The presented work presents protocols for HGMS of parasitized RBCs that fulfil these aims. Late-stage parasitized cell can be isolated essentially devoid of contamination with non-infected and ring-stage infected cells. Such an easy method for a highly quantitative and qualitative purification has not yet been reported. Synchronous cultures can be obtained both following depletion of late-stage infected cells, and following isolation of the latter. The quality of synchronization cultures matches that of sorbitol lysis, the current standard method for malaria culture synchronization. An advantage of HGMS is the avoidance of osmotic stress for RBCs. The new methods further have the appeal of high reproducibility, cost-effectiveness, and simple protocol.

It should be possible to take the methods beyond *Plasmodium* infected RBCs. Most magnetic separation techniques in the sector of biomedical research employ columns with a hydrophilic polymer-coated matrix. Our procedure employs an optimized buffer system. Polymer coating becomes unnecessary and uncoated columns are available at a fraction of the cost.

8 Zusammenfassung

Das Ziel dieser Arbeit war die Entwicklung von Aufreinigungsverfahren für *Plasmodium falciparum* infizierte Erythrozyten mittels Hochgradientenmagnetseperation (HGMS). Eine mögliche Anwendung dieser Verfahren sollte die Synchronisation von Zellkulturen sein.

Diese Arbeit bietet eine umfassende Beschreibung möglicher Anwendungen der HGMS, indem sie sowohl Depletion als auch Anreicherung beschreibt. Das Anreicherungsprotokoll erreicht Aufreinigungsraten von über 99 % spätinfizierter Erythrozyten. Eine vergleichbare Methode zur Aufreinigung der Zellen in dieser Quantität und Qualität ist uns nicht bekannt. Die beiden Verfahren konnten erfolgreich zur Synchronisation von Parasiten-Kulturen eingesetzt werden. Nach der Synchronisation entwickelten sich die Kulturen vergleichbar wie nach Synchronisation durch Sorbitol Lyse, dem aktuellen Standardverfahren. Auf HGMS beruhende Methoden haben den Vorteil, dass die Erythrozyten keinem osmotischen Stress ausgesetzt werden. Außerdem überzeugen die Protokolle durch eine hohe Reproduzierbarkeit, ein gutes Preis-Leistungs-Verhältnis und eine einfache Anwendbarkeit.

Die meisten magnetischen Separationsverfahren in der biomedizinischen Forschung arbeiten mit einer hydrophilen, polymerausgekleideten Matrix in den Separationssäulen. Unsere Methode bedient sich einer optimierten Pufferlösung. Dies macht die Polymerbeschichtung überflüssig und geht mit einer enormen Einsparung von Produktionskosten einher. Die Perspektiven für die zukünftige Anwendung des HGMS-Verfahrens in der Malaria-Forschung wurden aufgezeit. Die hier beschriebenen Protokolle können direkt in Diagnostik und Therapie angewendet werden.

9 References

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9.2 Electronic sources

1 WHO *Global Malaria Risk Areas* http://gamapserver.who.int/mapLibrary/Files/Maps/Global_Malaria_RiskAreas_2006.png. (24 April 2009).

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