# HAEMOGLOBIN UPTAKE IN RING STAGE PARASITE-INFECTED ERYTHROCYTES CONTAINING A FLUORESCENT ENDOCYTIC MARKER, TMR-DEXTRAN

by

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# LIST OF SYMBOLS, ABBREVIATION AND ACRONYMS

%..... percentage
°C..... degree celcius
~..... equivalence
ε.....extinction coefficient
≥ ..... more than or equal
μ..... micro
μL.....microliter
μM.....micromolar
3-D..... three-dimensional
A.....absorbance
c.....concentration
CCM.....complete culture medium
CLSM.....confocal laser scanning microscope
cm.....centimeter
$CO_2$.....carbon dioxide
DIC.....differential interference contrast
FZ.....food vacuole
*g* ..... g-force
Hb.....haemoglobin
Hz.....haemozoin
ICCM..... incomplete culture medium
kDa.....kilo-dalton
L..... litre
*l*..... length
LP.....long pass
M..... molar
m..... metre
$m^3$..... metre cube
mg..... milligram
Mg-ATP..... magnesium-adenosine triphosphate
mL..... milliliter

mM..... millimolar

mOsm/kg..... miliosmol per kilogram

NaCl..... sodium chloride

nm..... nanometre

P..... probability

*P. knowlesi*..... *Plasmodium knowlesi*

*P. falciparum*..... *Plasmodium falciparum*

*P. malariae*..... *Plasmodium malariae*

*P. ovale*..... *Plasmodium ovale*

*P. vivax*..... *Plasmodium vivax*

PBS..... phosphate buffer saline

PPM..... parasite plasma membrane

PPSK..... Pusat Pengajian Sains Kesihatan

PPSP..... Pusat Pengajian Sains Perubatan

PVM..... parasitophorous vacuolar membrane

RBC..... red blood cell

RBCM..... red blood cell membrane

rpm..... revolution per minute

RPMI medium..... Roswell Park Memorial Institute medium

SEM..... scanning electron microscopy

SD..... standard deviation

TMR-dextran..... tetramethylrhodamine-dextran

w/v..... weight per volume

WHO..... world health organization

# ABSTRACT

Early studies of the endocytic process in malaria parasite-infected erythrocytes showed that uptake of the host erythrocyte cytoplasm is most active in mature stage parasites (trophozoites). The parasite was thought to feed by distinct endocytic structures termed cytostomes. Vesicles that bud from the cytostomes were proposed to migrate to and fuse with the digestive vacuole where haemoglobin digestion and haem detoxification were thought to take place. Recent studies using serial thin-section electron microscopy however have led to conflicting conclusions. Hence, the aim of this work was to re-examine the endocytic process of live and intact parasites under non-disruptive conditions using live cell imaging by confocal microscopy. Resealed erythrocytes containing an endocytic marker, TMR-dextran were prepared using an optimised ratio of erythrocyte to haemolysis buffer volume (1:3) to minimise loss of haemoglobin while achieving a relatively homogenous population. TMR-dextran labelled erythrocytes were invaded by the parasites with similar efficiency to normal erythrocytes. Young stage parasites (rings) based on the estimated time after invasion and the absence of haemozoin showed punctate structures containing TMR-dextran near the parasite periphery suggesting that they probably represent early endocytic events.

# ABSTRAK

Kajian awal proses endositik dalam eritrosit yang dijangkiti parasit malaria menunjukkan pengambilan sitoplasma eritrosit adalah paling aktif ketika peringkat matang parasit (trofozoit). Sitostom yang merupakan struktur endositik yang unik dikatakan terlibat dalam proses pemakanan parasit. Kajian ini juga mendapati bahawa vesikel yang terhasil daripada sitostom bergerak dan kemudiannya bercantum dengan vakuol pencernaan yang merupakan tempat pencernaan hemoglobin dan penyahtoksikan hem berlaku. Bagaimanpun, kajian terkini menggunakan mikroskop elektron dan siri-siri keratan nipis menunjukkan keputusan kajian yang bercanggah. Oleh itu, matlamat kajian ini adalah untuk mengkaji semula proses endositik parasit yang hidup dalam persekitaran semulajadi dan tidak terganggu. Teknik pengimejan sel hidup menggunakan mikroskop konfocal telah digunakan. Eritrosit yang mengandungi indikator proses endositik yang berpendafluor, TMR-dextran disediakan menggunakan nisbah 1 isipadu eritrosit kepada 3 isipadu penimbal hemolisis bagi mengurangkan kehilangan hemoglobin dalam masa yang sama mendapat populasi sel yang homogen. Seperti yang berlaku pada eritrosit normal dan tanpa TMR-dextran, malaria parasit menjangkiti eritrosit mengandui TMR-dextran dengan efisien. Parasit peringkat awal (berbentuk cincin, berdasarkan anggaran masa selepas inokulasi dan ketiadaan hemozoin) memperlihatkan struktur kecil yang mengandungi TMR-dextran berdekatan periferi parasit. Ini menunjukkan kemungkinan berlakunya proses endositik di peringkat awal perkembangan parasit malaria.

# Chapter 1

# Introduction

## 1.1 Background of the study

Malaria is a mosquito-borne infectious disease of humans and other animals. It is caused by protozoan parasites of the genus *Plasmodium.* The malaria parasites are transmitted by the bites of female *Anopheles* mosquitoes during blood meals. *P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi* are the five types of *Plasmodium* species that can cause malaria to humans. World Health Organization (WHO) reported that in 2013 there were over 40% of the world's population at risk of malaria infection (WHO, 2014). Globally, *P. falciparum* infections have been described as the most clinically significant infections due to its association with the high mortality and morbidity especially in the regions of sub-Saharan Africa (Anstey et al., 2009). In Malaysia, *P. falciparum* is the second *Plasmodium* species that causes the most serious form of disease after *P. knowlesi* (Ministry of Health Malaysia, 2013).

*P. falciparum* has a complex life cycle. It includes a sexual stage occurred in a mosquito vector and an asexual stage in human host. The asexual stage can be divided into an exoerythrocytic stage (occurred in the liver) and an intraerythrocytic stage (in the erythrocyte). In the intraerythrocytic stage, *P. falciparum* cyclically invades and proliferates in erythrocytes. This cycle is completed every 48 hours. While inside the erythrocyte, the malaria parasite takes up a large quantity of the host erythrocyte cytoplasm by a process known as endocytosis. The erythrocyte cytoplasm is delivered to the digestive vacuole through endocytic vesicles. This endocytosis process is important in order to support parasite growth and asexual replication during the intraerythrocytic stage (Lazarus et al., 2008).

An early study of the feeding process in malaria parasite-infected erythrocytes showed that haemoglobin uptake is the most active in trophozoite stage parasites involving a cytostomal system (Bannister et al., 2000). Cytostomes are formed by the invaginations of the parasite plasma membrane (PPM) and the parasitophorous vacuolar membrane (PVM). Double membrane-bound vesicles forming from the budding of the cytostomes migrate and fuse with the digestive vacuole (Francis et al., 1997).

Recent studies have re-examined the process of haemoglobin uptake in infected erythrocytes, however have shown conflicting conclusions. A study using serial thin-section electron microscopy proposed four different pathways for haemoglobin uptake by *P. falciparum*, which involve the "big bulp" formation, cytostome-derived vesicles and tubules, and phagotroph (Elliott et al., 2008). Another study that also examined the ultrastructure of the endocytic apparatus concluded that haemoglobin uptake occurs via a vesicle-independent process (Lazarus et al., 2008). Meanwhile, a study using live cell imaging technique described the digestive vacuole is formed by cytostome-derived vesicles in the late ring stage of parasite development (Abu Bakar et al., 2010). They also described that longer-lived extra-digestive vacuole structure could form in some cases.

Imaging of endocytosis of erythrocyte cytoplasm in *P. falciparum* may find a solution to some debate regarding the feeding process of the parasite. The results will help in the understanding of the precise mechanism of action and intracellular effect of important antimalarials. This study will have an implication for the action of artemisinin and quinolone, which their modes of action in each case depend on the digestion of endocytosed haemoglobin in the parasite digestive vacuole.

## 1.2 Objectives of the study

### 1.2.1 General objective

To monitor the haemoglobin endocytosis of *P. falciparum*-infected erythrocytes

### 1.2.2 Specific objectives

i. To set up the *in vitro* culture of *P. falciparum*

ii. To prepare resealed erythrocytes containing a fluorescent probe, TMR-dextran

iii. To culture *P. falciparum* with resealed erythrocytes containing tetramethylrhodamine (TMR) -dextran

iv. To observe the haemoglobin endocytosis in *P. falciparum*-infected erythrocytes resealed to contain TMR-dextran by confocal microscopy

# Chapter 2

# Literature review

## 2.1 Malaria overview

According to WHO, more than 3.3 billion people were at risk of malaria infection and approximately 584 000 died due to malaria in 2013 (WHO, 2014). Most of the death cases were reported among African children. In Malaysia, there were 4 725 malaria cases with 16 deaths reported in 2012 (Ministry of Health Malaysia, 2013). *P. falciparum* is the second *Plasmodium* species that can cause the most serious form of disease in Malaysia after *P. knowlesi.* Globally, *P. falciparum* infections have often been seen as the most clinically significant infections due to their association with mortality and the intensity of infections in sub-Saharan Africa (Anstey et al., 2009).

Malaria can be categorised as uncomplicated and severe infection based on the symptoms shown. Common symptoms of uncomplicated malaria cases are relapsing fever, chills, head and body aches, nausea and vomiting (Abdallah et al., 2013). Some of these characteristic symptoms such as relapsing fever and body ache are caused by the cyclic release of haemozoin and bilirubin as the infected erythrocytes burst. For severe malaria, the clinical manifestation includes severe anaemia, cerebral malaria and respiratory distress.

## 2.2 Life cycle of *P. falciparum*

*P. falciparum* undergoes its life cycle inside a human host and a female *Anopheles* mosquito.

### 2.2.1 Sporogonic cycle of the parasite in a mosquito

Ingestion of gametocytes by the mosquito from an infected human induces the sporogonic cycle. Factors inducing gametogenesis include a drop in temperature, and an increase in carbon dioxide and mosquito metabolites. Microgametes formed through a process known as exflagellation fertilise macrogametes forming zygotes (see Step 1, Figure 2.1). The zygotes develop into motile ookinetes, which penetrate the gut epithelial cells and further develop into oocysts (Step 2, Figure 2.1). The oocysts undergo the asexual replication producing sporozoites (Step 3, Figure 2.1). The oocysts rupture and release sporozoites into the haemocoel (a body cavity) of the mosquito. The sporozoites invade the salivary glands, hence completing the life cycle inside the mosquito host (Da Silva, 1999).

### 2.2.2 Schizogony of the parasite in a human host

The parasites are transmitted to humans by injection of sporozoites into the bloodstream by infected female *Anopheles* mosquitoes during a blood meal. They undergo the extraerythrocytic (in the liver) & intraerythrocytic cycles (in the erythrocytes).

#### 2.2.2.1 Exoerythrocytic cycle of the parasite

The sporozoites migrate to the liver and invade hepatocytes to begin the exoerythrocytic cycle (Step 5, Figure 2.1). Inside the hepatocytes, the parasites undergo an asexual replication known as an exoerythrocytic schizogony (Step 6, Figure 2.1) (Mota et al., 2002). The parasites grow to mature schizonts rupture and release merozoites into the bloodstream (Step 7, Figure 2.1).

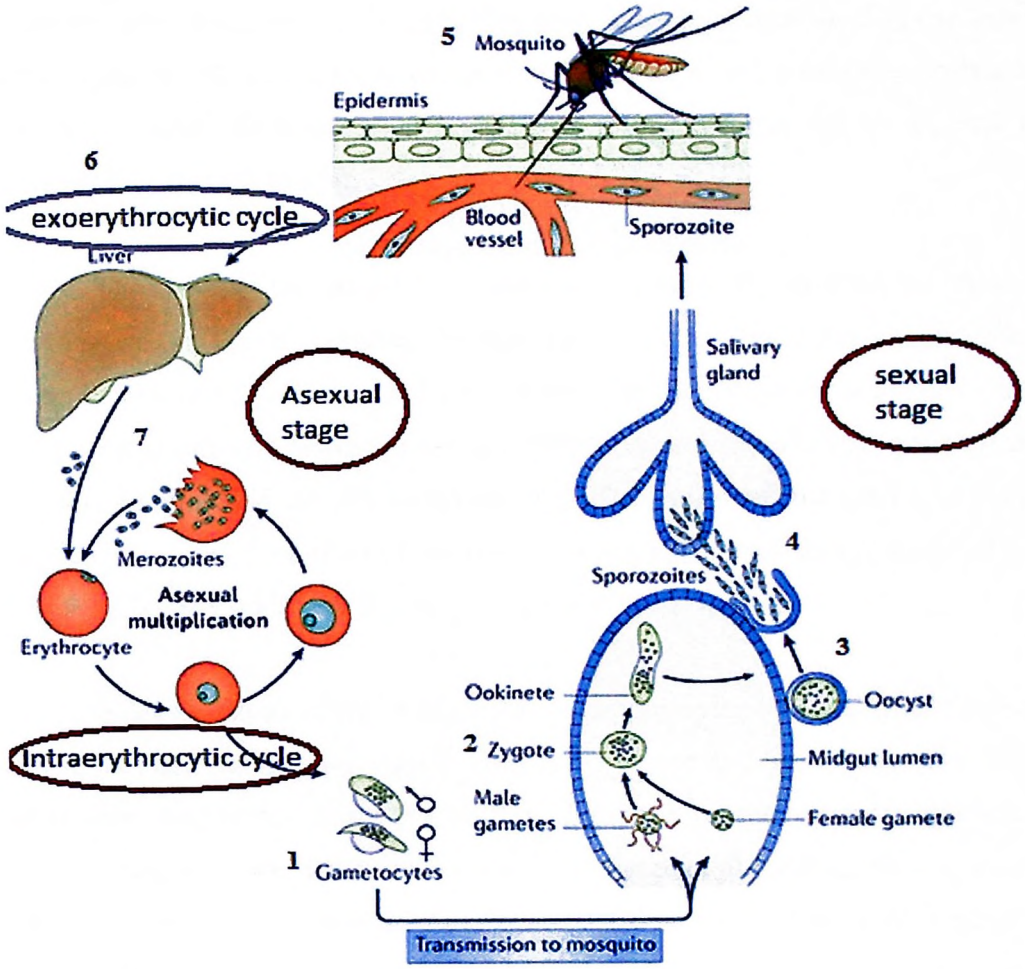



**Figure 2.1: The life cycle of the malaria parasite, *P. falciparum***

(Step 1) Microgamete and macrogamete fertilise to form a zygote. (Step 2) A zygote develops into a motile ookinete and further grows into an oocyst. (Step 3) The oocyst grows and multiplies producing many sporozoites. (Step 4) The oocyst ruptures and releases sporozoites into haemocoel of the mosquito. (Step 5) The sporozoites are injected with saliva during blood feeding of the mosquito. (Step 6) Inside the human host, the parasite undergoes an exoerythrocytic schizogony inside the hepatocyte producing many merozoites. (Step 7) The merozoites enter the blood stream and invade erythrocytes initiating an intraerythrocytic cycle (Bousema et al., 2014).

### 2.2.2.2 Intraerythrocytic cycle of the parasite

The merozoites formed during the exoerythrocytic cycle are carried by the blood stream and invade erythrocytes. They undergo a cycle of asexual division that last for approximately 48 hours. Once completed, it releases 10-24 new merozoites into the vascular system (Reagen, 2006). The pathology of malaria is caused by this intraerythrocytic cycle of the parasite.

The onset of the intraerythrocytic cycle is when the merozoites released from the hepatocyte rupture, entering the blood stream and invade the host's erythrocytes (Step 1, Figure 2.2). Merozoites have lemon-like shape, ovoid with low, flat ended projection at one end (Bannister et al., 2000). Merozoites of *P. falciparum* probably are the smallest of all *Plasmodium* spp. The apex of merozoites contains the secretory vesicles, a number of ribosomes, a mitochondrion and plastid. The nucleus is located basally and there is less cytoskeleton.

After invasion of the merozoites in erythrocytes, the parasite flattens into a thin discoidal, flat or cup-shaped ring form (Step 2, Figure 2.2). The ring of *P. falciparum* has width less than half of diameter of the nucleus. It contains 1-2 nucleus, round at one site of cytoplasm (Bannister et al., 2000). Appearances of the nucleus differ, from a sausage-like form to a disc form. The central region of the parasite is quite thin while the peripheral region is thicker. Major organelles including nucleus, mitochondrion, plastid, most of the ribosomes and endoplasmic reticulum (ER) are placed in the thicker region of cytoplasm, while the central region of the disc contains few structure. A group of vesicles, smooth and rough ER, and a small Golgi body are located near to the nucleus. The ring stage takes about half of the intraerythrocytic cycle, which is about 20 hours duration. The ring changes shape to a rounded or irregular trophozoite (Step 3, Figure 2.2). Difference between the ring and trophozoite stages are mainly on cell size and shape.

As the trophozoite matures, it transforms to a multinucleated schizont (Step 4, Figure 2.2). The schizont is the final stage in the intraerythrocytic cycle and releases merozoites that can invade new uninfected erythrocytes. The schizont undergoes four

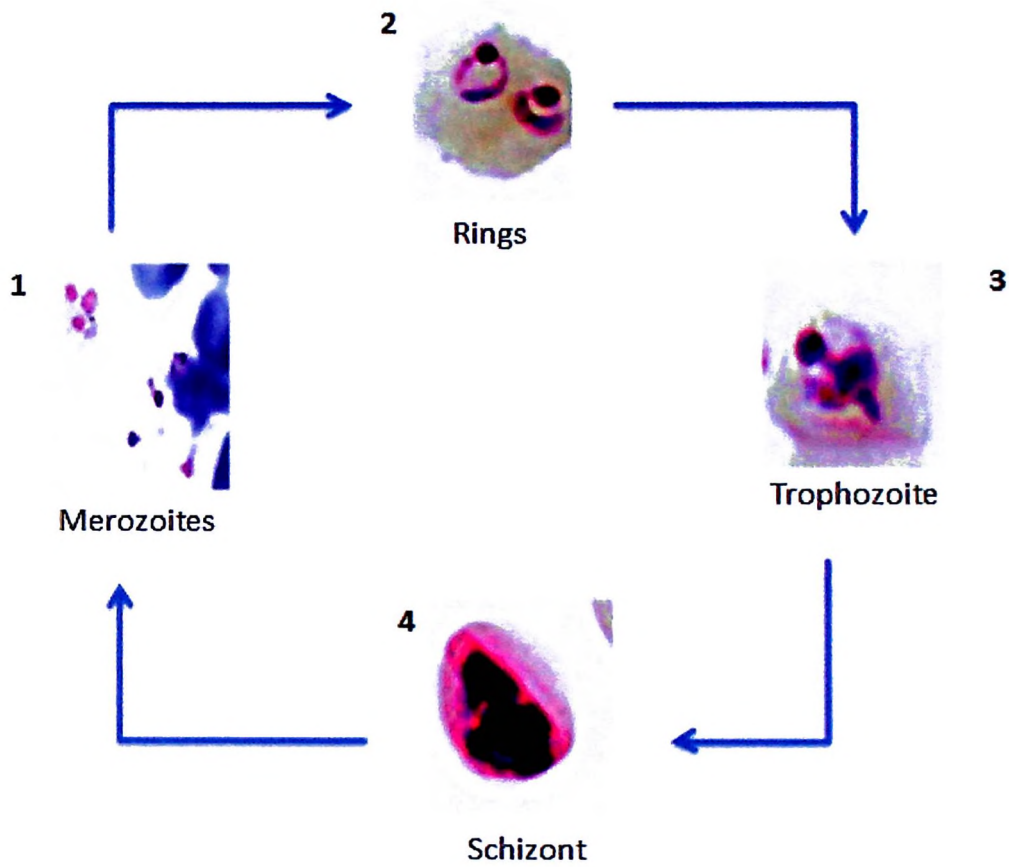



**Figure 2.2: The intraerythrocytic stages of *P. falciparum***

(Step 1) It starts when hepatic merozoites released from the hepatocyte rupture enter the blood stream and invade the host's erythrocytes. (Step 2) After invasion, a ring-shaped parasite is formed. (Step 3) The parasite matures into a trophozoite. (Step 4) The trophozoite further develops into a multinucleated schizont. Erythrocytic merozoites are released from the erythrocyte rupture, which will invade new erythrocytes to repeat the blood cycle (Shahinas et al., 2013).

round of nuclear division in order to produce about 16-20 merozoites (Bannister et al., 2000).

The intraerythrocytic cycle produces more merozoites after repeated periodic rupturing of erythrocytes (Crutcher & Hoffman, 1996). The life cycle is completed when a feeding mosquito takes a blood meal containing male and female gametocytes that are capable of undergoing sexual development. Analysis of the blood stages is made possible by the availability of an *in vitro* method for the cultivation of the intraerythrocytic stages of *P. falciparum* (Trager & Jensen, 1976).

## 2.3 Endocytosis in general

Endocytosis is a collective term refers to many pathways through which a variety of molecules can be internalised into cells (Pryor & Luzio, 2008). For example, extracellular material and plasma membrane proteins are delivered to lysosomes for degradation via the endocytic pathway. This endosomal-lysosomal pathway is used by many organisms for ingestion of essential nutrients, removal of dead or damaged cells from the body and defense against microorganisms. Specifically, the formation of lysosome occurs through the binding of transport vesicles budded from the trans Golgi network with endosomes, which contain molecules taken up by endocytosis at the plasma membrane (Mukherjee et al., 1997).

### 2.3.1 The role of endocytosis in the malaria parasite

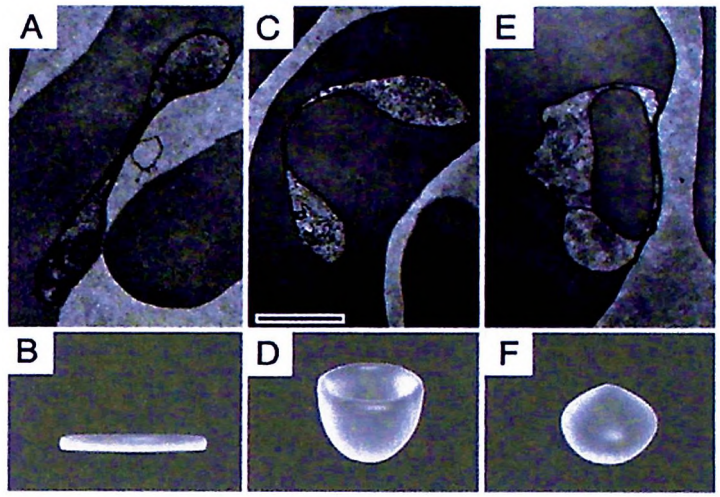
The malaria parasite undergoes endocytosis in order to internalise the host cell cytoplasm. This process supports parasite growth and asexual replication during the intraerythrocytic cycle. The endocytosis of the erythrocyte cytoplasm by the parasite is thought to be initiated by cytostomes (Hanssen et al., 2010). Inside the erythrocyte, the parasite is surrounded by three different membranes, the parasitophorous vacuolar membrane (PVM), the parasite plasma membrane (PPM) and the erythrocyte membrane. The cytostome is a pear shaped large double membrane structure (Francis et al., 1997). Outer membrane of cytostomes is derived from the PPM and the inner membrane from the PVM (Elliot et al., 2008). When the

cytostome ingest cytoplasm of erythrocytes, double membrane vesicles are formed by budding (Francis et al., 1997). The vesicles containing mainly haemoglobin are transported to an acidic digestive vacuole. The free haem resulting from haemoglobin digestion is converted into inert black-brown pigment crystals known as haemozoin that accumulate within the digestive vacuole (Dasari et al., 2012).

The mechanism responsible for the transport of host cell haemoglobin to the digestive vacuole of the parasite is still debated. Recently, a study using serial thin-section electron microscopy and three-dimensional reconstruction have revealed four different pathways for haemoglobin uptake by the malaria parasite (Elliot *et al.*, 2008). They suggested a "big gulp" formation at the early ring stage parasites (Figure 2.3 i). The parasites underwent morphological changes in which they fold like a cup and take a large first gulp of host cell cytoplasm. The second structure was similar to the classical endocytic structure, cytostome, giving rise to small vesicles and tubules (Figure 2.3 ii). Finally was a phagotroph, which is similar to the "big gulp" (Figure 2.3 iii).

Another study by Lazarus et al. (2008) suggested that haemoglobin transport to the digestive vacuole occurs via a vesicle-independent process (see Figure 2.4). Instead of the formation of vesicles, the cytostomal invaginations elongate to form tubes. They fused with the digestive vacuole membrane at the same time as they bud off from the parasite surface.

Study by Abu Bakar et al. (2010) based on a combination of electron tomography and live cell imaging described that haemoglobin digestion and haemozoin formation took place in cytostome-derived vesicles. Cytostomes were active as early as in early ring to mid ring stage (Figure 2.5A). The ring stage parasite could formed a deeply invaginated cup shape but do not took up haemoglobin by micropinocytosis (Figure 2.5B). The cytostome-derived vesicles formed the digestive vacuole in the late ring stage of parasite. As the parasite matures, cytostomal invaginations continue formed and budded into parasite cytoplasm where they are rapidly acidified (Figure 2.5C). Other than cytostome-derived vesicles, cytostome-independent budding of the parasite surface may lead to the uptake of the host cytoplasm. These structures could form longer-lived extra-




i) The "big gulp" formation

ii) Cytostome-derived tubule

iii) Phagotroph

**Figure 2.3: Distinct pathway of haemoglobin uptake by the malaria parasite**

(i) The "big gulp" formation at the early ring stage parasites. (ii) Cytostome that gives rise to tubules and vesicles. (iii) Cytostome-independent phagotroph forms (Elliot et al., 2008).

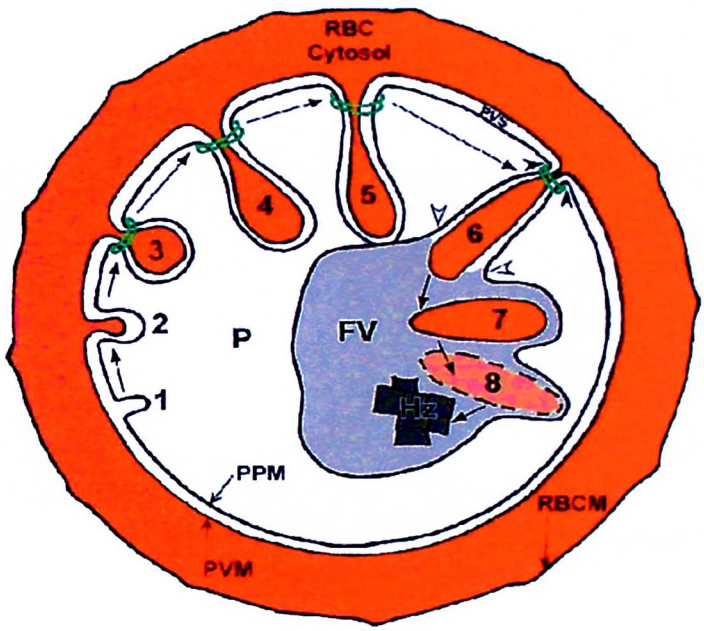



**Figure 2.4: Vesicles-independent process of haemoglobin uptake by the malaria parasite**

Instead of formation of vesicles, the cytostomal invaginations elongate to form tubes. They fuse with the digestive vacuole membrane at the same time as they bud off from the parasite surface (Lazarus et al., 2008).

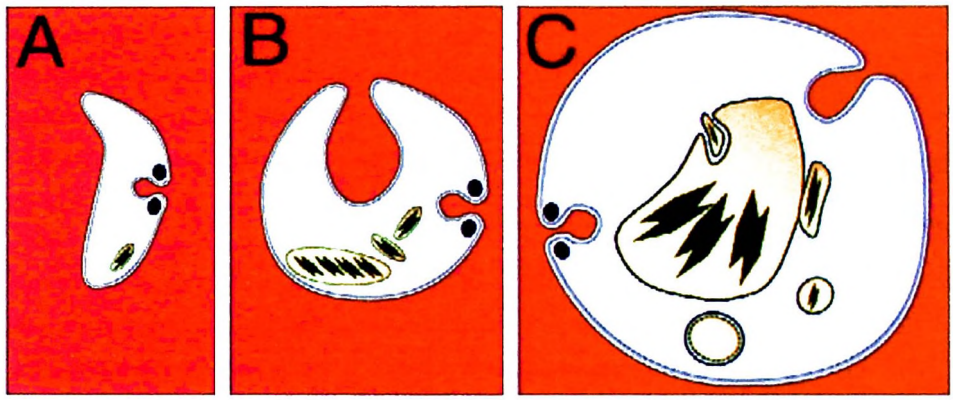



**Figure 2.5: Endocytic processes and digestive vacuole genesis in the malaria parasite**

(A) Cytostomes become active in early ring to mid ring stage. (B) The ring stage parasite forms a deeply invaginated cup shape but does not take up haemoglobin. (C) The cytostome-derived vesicles form the digestive vacuole in the late ring stage of the parasite. As the parasite matures, cytostomal invaginations continue to form and bud into the parasite cytoplasm where they are rapidly acidified (Abu Bakar et al., 2010).

digestive vacuole structures where haemoglobin digestion was formed first before they fused with the digestive vacuole.

Hence, there is a need to re-assess the endocytic process since it plays an important role in some antimalarial activity. In this study, the endocytic process was re-assess by using Confocal Laser Scanning Microscopy (CLSM), which image the haemoglobin endocytosis of the parasite in resealed erythrocytes containing tetramethylrhodamine (TMR)-dextran.

## 2.4 Methods of entrapping high molecular weight endocytic markers

Methods for trapping high molecular weight dextrans that linked to fluorescent markers in resealed erythrocytes have been developed (Krogstad, *et al.*, 1985). Erythrocytes resealed to contain dextran conjugates are prepared by collecting blood samples, separating erythrocytes from plasma, entrapping dextran-linked fluorescent markers in the erythrocytes and resealing the erythrocytes. In this study, tetramethylrhodamine (TMR), that conjugates to dextran was used as an endocytic marker.

Dextrans are hydrophilic polysaccharides distinguished by their moderate to high molecular weight, good water solubility and low toxicity (Molecular probes, 2006). Labelled dextrans are hydrophilic polysaccharides that are most commonly used in microscopy studies to monitor endocytosis, cell division and movement of live cells. The labelled dextrans are inserted into erythrocytes before parasite invasion. After invasion, the probes are taken up along with the erythrocytes haemoglobin. Hence, it can be used to follow endocytic processes by fluorescence microscopy.

## 2.5 Imaging of parasite-infected resealed erythrocytes

Confocal laser scanning microscopy (CLSM) has been extensively employed in cell imaging and other biological applications since early 1980s (Claxton et al., 2006). It

gives much better spatial resolution of three-dimensional structures compared to traditional fluorescence microscopy, producing images free from out-of-focus blur. This optical set-up offers great flexibility in image acquisition strategies. It commonly employs visible wavelength lasers as light sources and confocal apertures or "pinholes" in the detection path to exclude out of focus light. It can generate diffraction-limited, and, in nonlinear versions, sub-diffraction limited 3-D images of microscopic samples and, for example, localize multiple fluorescent labels in 3-D space within a single sample. Confocal Laser Scanning Microscopy (CLSM) is also preferred over Scanning Electron Microscope (SEM), which usually requires intensive sample pre-treatment. Another distinguishing advantage of CLSM is that it allows the optical sectioning of a 3-D object and subsequent 3-D visualisation using 3-D rendering software. CLSM has proven to be an extremely versatile tool and has been used for the study of cells and cellular event.

# Chapter 3

# Materials and Methods

## 3.1 Parasite culturing methods

### 3.1.1 Parasite strains

3D7 chloroquine-sensitive strains of *P. falciparum* acquired from Institute for Research in Molecular Medicine (INFORMM) were used in this study.

### 3.1.2 *In vitro* culturing of parasite-infected erythrocytes

Parasites were grown in culture flasks containing complete culture medium (CCM) using a method modified from Trager and Jensen (1976). Washed O+ type human erythrocytes acquired from Transfusion Unit of Hospital Universiti Sains Malaysia (HUSM) were used and maintained at 3-5% haematocrit using a method modified from Trager and Jensen (1976). The CCM consists of 500 mL RPMI 1640 (Sigma, R-0883), 5 mL of 20% glucose, 3 mL 1M sodium hydroxide, 1.25 mL of gentamicin (Sigma), 5 mL 200mM glutamine (Sigma) and 40 mL of human serum. The medium was changed once every two days. Parasite cultures were grown in an incubator kept at 37°C. The gas mixture contains 1% oxygen, 3% carbon dioxide and 96% nitrogen. The assessment of parasitaemia and parasite stage was done daily by microscope examination of thin blood smears stained with Giemsa stain.

### 3.1.3 Synchronisation of ring stage parasite-infected erythrocytes

Synchronisation of ring stage parasites was performed by using a protocol modified from that of Lambros and Vanderberg (1979). It was done by selective lysis of mature stage parasites. The optimum condition for synchronisation was when the parasites were at the ring stage with more than 5% parasitaemia. The cultures of the

parasites were transferred to sterile 50 mL Falcon tubes and pelleted by centrifugation (1500 rpm, 5 minutes). The pellets were suspended with 10 cell pellet volumes of 5% D-sorbitol (Sigma) in water and left for 10 minutes in room temperature. The samples were centrifuged (1500 rpm, 5 minutes) and the pellets were resuspended in CCM. The parasites were incubated at 37°C under normal culture conditions for at least 2 hours before being used.

### 3.1.4 Purification of mature stage parasite-infected erythrocytes

Purification using a Percoll density gradient was performed by using a method established by Knight and Sinden (1982). Mature stage parasites (trophozoites or schizonts) were harvested when the parasite cultures at ≥ 5% parasitaemia. The cultures of the parasites were transferred to 50 mL sterile Falcon tubes and centrifuged at 1500 rpm for 5 minutes. Supernatants were discarded and pellets were slowly pipetted on top of 35/65 % Percoll (w/v) (Amersham Pharmacia) then centrifuged (2500 rpm, 15 minutes, no brakes). Purified mature stage parasite-infected erythrocytes, which were identified by a dark grey middle layer appearance were transferred to a sterile 10 mL tube and washed 3 times with incomplete culture medium by centrifugation (1500 rpm, 5 minutes). The pellets were collected and an aliquot (~0.5 µL) of the pellets was used to determine the parasitaemia and parasite stage by preparing Giemsa-stained thin blood smears.

### 3.1.5 Optimisation of Giemsa staining

Giemsa stain procedure was optimised by preparing three different concentrations (10, 15 and 20%) of Giemsa stain diluted with 0.5x PBS (pH 7.2). Thin blood smears were stained with respective concentrations of Giemsa solution for 5, 10, 15 and 20 minutes after fixation in 100% methanol (approximately 15 seconds). The Giemsa stain concentration of choice for demonstrating rapidly and clearly the presence of parasites in thin blood smears was selected and used throughout the experiments.

## 3.2 Analysis of resealed erythrocytes

### 3.2.1 Preparation of fluorescent probe stock solutions

25 mg of tetramethylrhodamine-dextran (TMR-dextran, 10 kDa, Invitrogen) were dissolved in 1 mL haemolysis buffer (5 mM sodium phosphate buffer, pH 7.5). The TMR-dextran concentrations in haemolysis buffer was measured spectrophotometrically at 543 nm using the molar extinction coefficient (ε) value of 99 mM-1cm-1, as provided by the manufacturer. 29.41 µL of the primary stock solution was aliquoted in microfuge tubes, wrapped in foil and kept at -20°C.

### 3.2.2 Resealing method

The optimum ratio of packed erythrocytes to haemolysis buffer volume that allow partial loss of haemoglobin while introducing the fluorescent probe into cells need to be determined first. A protocol for resealing of erythrocytes was modified from that of Dluzewski *et al.* (1983). Packed cells were washed twice with sodium phosphate buffer (20 mM, pH 7.5) by centrifugation (3000 rpm, 10 minutes) to remove Buffy coat. Three different ratios of packed erythrocyte to haemolysis buffer were chosen for this optimization test. The ratios were 1:2, 1:3 and 1:4. Aliquots of the washed cells were transferred to microfuge tubes and lysed in ice-cold haemolysis buffers. Tubes were agitated to ensure even haemolysis of the cells before incubated on ice for 10 minutes. Then sodium chloride (NaCl) was immediately added (from a stock of 5 M) to a final concentration of 0.15 M to reseal the cells. Tubes were incubated in orbital shaker at 37°C for 45 minutes. The total amount of haemoglobin in the samples was measured at this point and processed as explained in Section 3.2.3. After incubation, cells were centrifuged at 3000 *g* for 5 minutes. The supernatants were collected to measure the amount of haemoglobin loss after resealing (see section 3.2.3 for details). The pellets were further washed at least three times until a clear supernatant was obtained. Cell pellets were collected and the amount of haemoglobin retained in resealed erythrocytes was measured (see section 3.2.3 for details).

### 3.2.3 Measurement of haemoglobin loss and retention within resealed erythrocytes

The total amount of haemoglobin from the samples prepared as indicated in the previous section 3.2.2 was measured as follows: after the addition of NaCl, 40 µL aliquots of cell suspensions were subsequently removed from the samples, suspended in 110 µL haemolysis buffer and two-fold diluted in haemolysis buffer across 96-well microtiter plates. The total concentration of haemoglobin was measured on a microplate reader by reading the absorbance at 415 nm, and calculated using the ε of 50.1 mM-1cm-1. The amount of haemoglobin loss from, and retained within, resealed cells was measured as follows: after 45 minutes incubation in the presence of NaCl, cell suspensions were pelleted (3000 *g*, 5 minutes) and supernatants were collected to measure haemoglobin loss, and processed as mentioned above. Meanwhile, aliquots of resealed cells were suspended 1:1000 in haemolysis buffer. Then, 40 µL of the cell suspensions were resuspended in 110 µL haemolysis buffer, two-fold diluted across 96-well microtiter plates, and analysed as mentioned above. This assay was done in triplicate.

### 3.2.4 Preparation of resealed erythrocytes containing fluorescent probes

Using an optimised ratio of packed erythrocytes to haemolysis buffer volume, cells were lysed in 3 volumes of ice-cold haemolysis buffer containing 1 mM Mg-ATP in the presence of 50 µM TMR-dextran (Figure 3.1) (see section 3.2.2 for details). After resealing in NaCl, cell suspensions were pelleted (3000 rpm, 5 minutes) and washed with phosphate buffer saline (1x PBS, pH 7.5) until a clear supernatant was obtained. Pellets containing resealed cells were collected, resuspended in CCM, wrapped in foil, and kept at 4°C for subsequent use.

### 3.2.5 Culturing parasites in resealed erythrocytes

To start the inoculation of parasites inside resealed erythrocytes, synchronised mature stage parasites were harvested by floatation on a Percoll gradient consisting

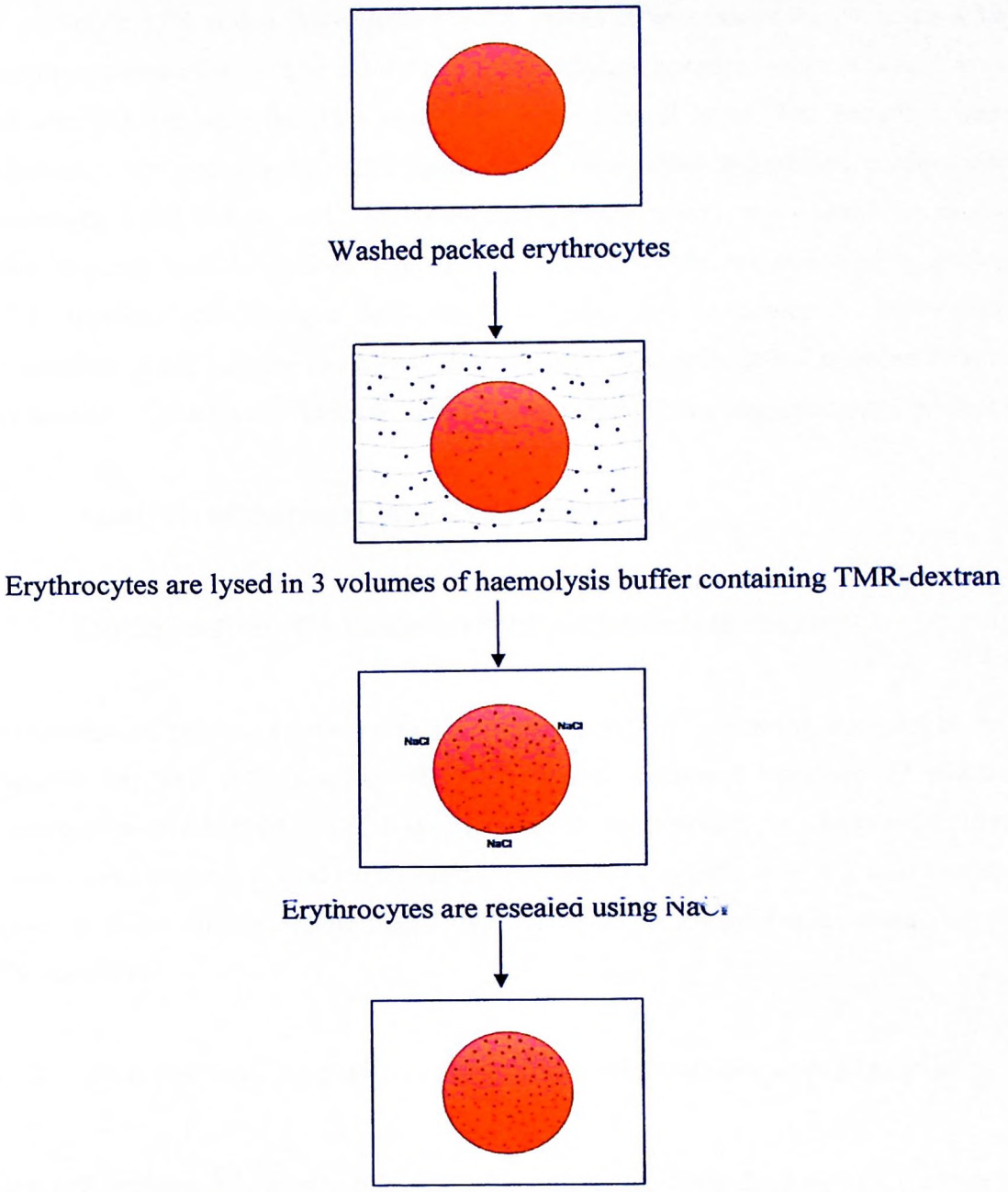



**Figure 3.1: Preparation of resealed erythrocytes containing a fluorescent probe, TMR-dextran**

Washed erythrocytes were lysed in ice-cold haemolysis buffer in the presence of TMR-dextran (30 μM). The cells were incubated on ice for 10 minutes. After incubation, the cells were placed in hypertonic solution of NaCl (a final concentration of 0.15 M to reseal the cells. The cells were incubated for 45 minutes at 37°C, washed 3 times with 1x PBS (pH 7.5), resuspended in CCM, wrapped in foil, and kept at 4°C for subsequent use.

of an upper 35% and a lower 65% Percoll layers at approximately 24 hours after synchronisation (see section 3.1.4 for details). Mature parasites were obtained from the interface between the 35% and 65% of the Percoll layer. The parasites were adjusted to 3% parasitaemia (2% haematocrit) then added to resealed erythrocytes containing TMR-dextran in CCM. Resealed erythrocytes with and without the probe, were cultured in 6-well plates under normal culture conditions indicated in section 3.1.2. Medium was changed daily. Invasion index was calculated 24 hours after inoculation of the parasite in order to determine the susceptibility of resealed cells to the parasite. The invasion index is defined as the ratio of ring stage parasites present

## 3.3 Analysis of parasite endocytic processes

### 3.3.1 Optimisation of haematocrit of packed erythrocytes

Percentage of packed erythrocytes (% haematocrit) for preparing monolayer cell cultures for live cell imaging was determined. Different volumes of packed erythrocytes (0.03, 0.04, 0.05, 0.06 and 0.07% haematocrit) in complete culture media were prepared and added to culture chambers. The cells were allowed to settle down for a few minutes before being observed under an inverted microscope using a 40x objective.

### 3.3.2 Live cell imaging of parasite-infected resealed erythrocytes

Live cell imaging of parasite-infected cells containing TMR-dextran was performed at 37°C using a 40x objective on an inverted confocal laser scanning microscope (see Figure 3.2) (Department of Neuroscience, PPSP). The microscope was equipped with an incubation chamber and a heated stage maintained under an atmosphere of 5% $CO_2$ in air (Figure 3.3). In this present study, a culture dish made as housing for a glass slide containing a sample was used. Infected resealed erythrocytes containing TMR-dextran were mounted on a glass coverslips using an optimised 0.05% haematocrit in cell suspension (see section 3.3.1 for details). The cell suspension was left for ~5 minutes to settle down before being imaged using the microscope. TMR-dextran was excited at 543 nm and the red fluorescence was detected using a 560 LP

filter set. Images collected were 8 bit images typically 256 by 256 pixels. Image analysis was performed using ImageJ software.

## 3.4 Statistical analysis

Statistical analysis was done by using IBM SPSS Statistic software.

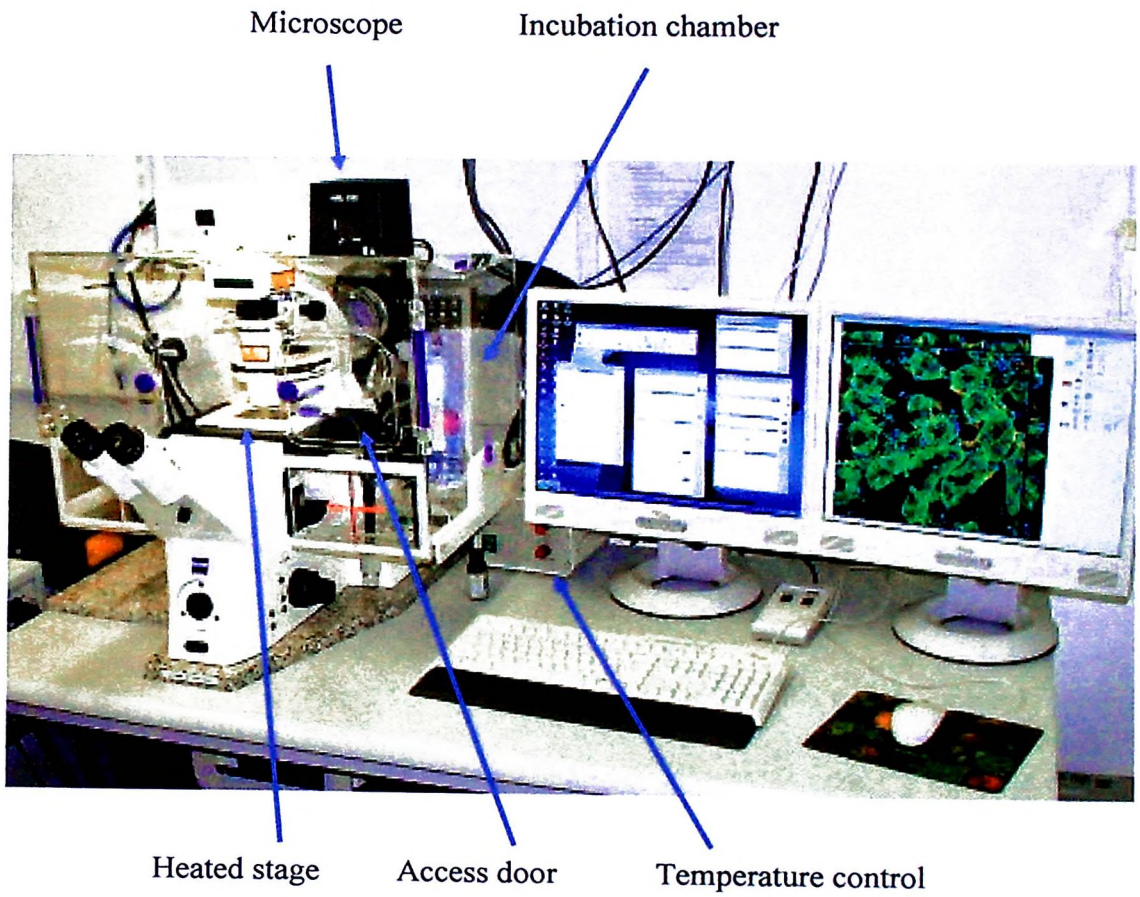



**Figure 3.2: Common components of confocal laser scanning microscope (Zeiss LSM 510)**

The confocal laser scanning microscope (Department of Neuroscience, PPSP) was used for life cell imaging of the malaria parasite's endocytic process. The microscope is equipped with incubation chamber and contains $CO_2$ gas supply in order to keep the cells alive.

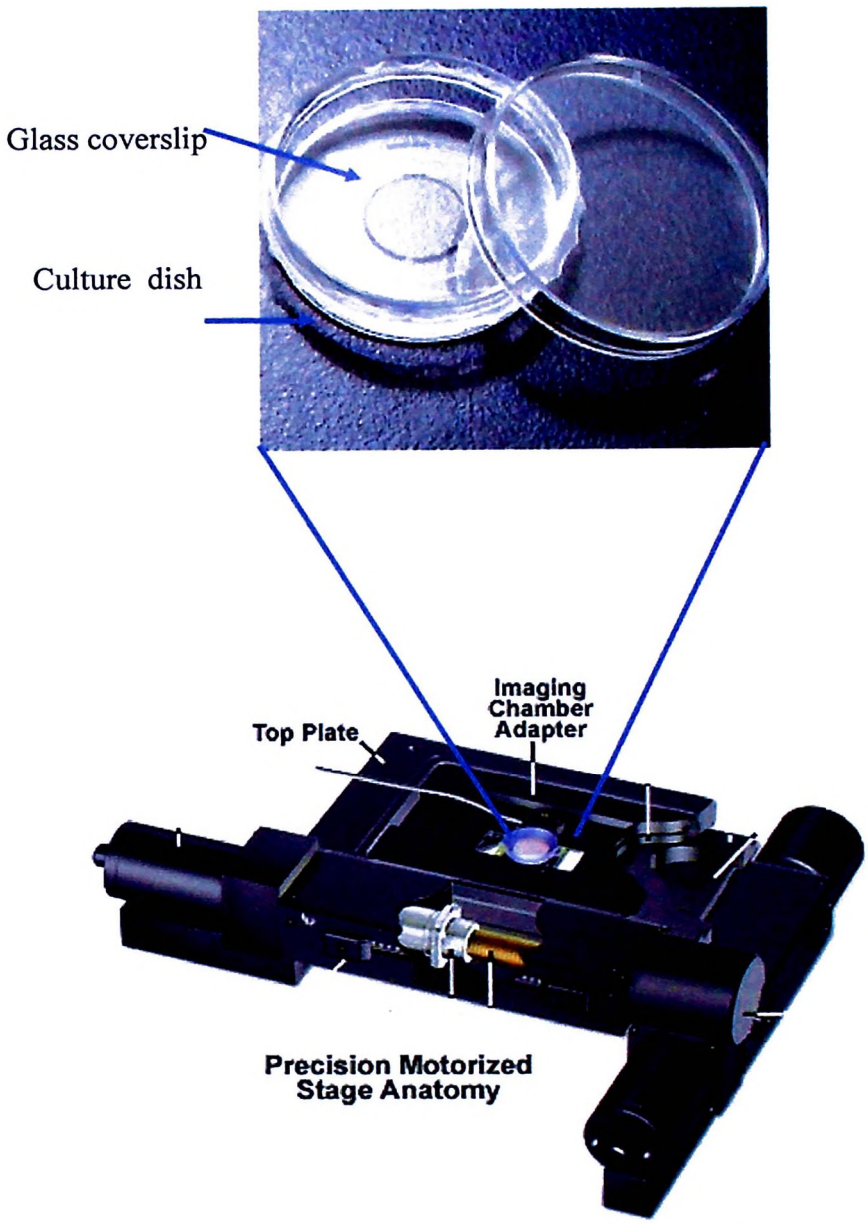



**Figure 3.3: Motorized stage of the confocal laser scanning microscope**

The confocal laser scanning microscope uses a high-performance motorized stage for eliminating the possibility of lateral stage drift and gathering sequential images of two or more viewfields during time-lapse sequence acquisition. In this study, the culture dish housing the glass slides was used to avoid any split of the sample.