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Tracking the Formation of Rhoptry Organelles and Parasitophorous Vacuole in *Plasmodium falciparum* Using the Green Fluorescent Protein

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Abstract: The most deadly Apicomplexan parasite is *Plasmodium falciparum* because it causes the globally spread human malaria especially in Africa. Like other members of Apicomplexa, *Plasmodium* is an intracellular parasite that uses an elaborate complex of apical secretory organelles (rhoptries, micronemes and dense granules) to invade its host cells and immediately accommodate itself in a parasitophorous vacuole (PV). In this study, we have cultured a previously established *P. falciparum* cell line that expresses the green fluorescent protein to visualize, *in vivo*, the formation of PV and rhoptry organelles. Parasites were tightly synchronized and investigated at short intervals and the formation of PV and rhoptry organelles were tracked in detail. Our results showed the development of circular extensions from PV in the asexual stages. Data showed that gametocytes develop very long tubular structures that run marginally underneath the erythrocyte cell membrane and reach the cell surface. Also, our data revealed the formation of crescent-like structures at the periphery of the immature schizonts, which were equivalent in number to and surrounding the nuclear lobes. These findings confirm the advantage of GFP use as a biomarker to track the formation of subcellular organelles and structures *in vivo*.

Key words: Rhoptries · Parasitophorous Vacuole · Organelles · Biogenesis · *Plasmodium* · Apicomplexa

INTRODUCTION

The phylum Apicomplexa includes a large number of obligate intracellular parasites. Among these are some notorious human and animal pathogens from genera such as *Plasmodium* that causes malaria, *Toxoplasma* which causes a congenital disease and infection in immune-compromised patients [1], *Cryptosporidium* which causes serious gastrointestinal disease and *Eimeria* that represents a major problem in the poultry industry and a cause of chicken coccidiosis. The phylum is defined by the presence of an apical complex that comprises a microtubule anchoring ring through which dedicated secretory organelles release their contents [2]. There are three types of apical secretory organelles-the small, rod-shaped micronemes, the much larger, bulb-shaped rhoptries and round dense granules.

For a successful cell invasion, members of the Apicomplexa, relying on their own motility, anchor themselves firmly to their host cell by secreting a group of specific proteins and establishing moving junction at the interaction surface of the host cells. The moving junction moves from the apical to the posterior end of the parasite, leading to the internalization of the parasite into a parasitophorous vacuole (PV). The parasite exports the microneme protein AMA1 to its own surface and the rhoptry neck RON2 protein as a receptor inserted into the host cell together with other RON partners [3]. All RON proteins are secreted from rhoptries and several RON proteins have been identified in *Toxoplasma* and *Plasmodium* and interestingly RON2, RON4, RON5 and AMA1 orthologues can be found in the genomes of *Plasmodium*, *Toxoplasma*, *Neospora*, *Eimeria*, *Theileria* and *Babesia* species. This suggests a ubiquitous and conserved function of the moving junction within these parasites [3]. Also, this highlights the importance of rhoptry organelles and the crucial role that rhoptry proteins play in the invasion process of host cells by a wide range of parasites.

Although difficult to observe by microscopy, the PV of *Plasmodium* is the subject of intense research. The PV is formed at the time of invading the host cell and is considered as convenient niche located at the interface between the parasite and its host cell and offers

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protection and nutrients to allow efficient parasite multiplication. Once inside the erythrocyte, the merozoite loses its apical secretory organelles and immediately starts to differentiate into ring, trophozoite and eventually schizont. Host cell lipids are largely incorporated into the forming parasitophorous vacuolar membrane (PVM) at the time of invasion [4]. The *Plasmodium* PVM was shown to incorporate many host cell components, like cholesterol, sphingolipids, GPI-anchored proteins and even integral membrane proteins, into cholesterol-rich, detergent resistant membrane domains (DRM or rafts) at the time of parasite invasion into the host cell [5-7]. The rhoptry protein RhopH2 [8] and the Pf stomatin (a major cholesterol binding protein, component of rafts in mammalian cells), which are mostly expressed at both the late trophozoite and schizont stages, are also shown to be associated to these PV rafts [9].

Fluorescent proteins are widely used as quantitative genetically encoded markers for studying protein-protein interaction, protein and cell tracking. Expression of chimeric proteins produced by fusing a fluorescent protein and the protein of interest (fusion protein) allows researchers to monitor target protein localization and to visualize dynamics of cell events [10]. In this study, we used a previously established *Plasmodium falciparum* cell line [11] that expresses the N-terminal third (amino acids 24-483) of the rhoptry protein Clag3.1 fused with the green fluorescent protein (GFP) to its C-terminus under the control of *PfRhopH2* promoter region. This Clag3.1-GFP chimera, under these control elements, localizes first to the parasitophorous vacuole and is then targeted to the merozoite rhoptries. Therefore, this study utilized this feature to track in detail the formation of the parasitophorous vacuole and rhoptries during the late developmental stages of *Plasmodium falciparum*.

MATERIALS AND METHODS

***In vitro* Culture of *P. falciparum*:** The Dd2 strain of *P. falciparum* was grown *in vitro* under the standard conditions [12] with little modifications. Briefly, incomplete medium (ICM) was prepared by dissolving 5.94 gm of HEPES and 0.05 gm of Hypoxanthine into 750 ml of deionized distilled water, then 1 pack of L-Gln-containing RPMI 1640 powder was added and the solution was brought to 1L. Complete medium (CM) was prepared by mixing 450 ml of ICM, 15 ml of Sodium Bicarbonate (7.5%) and 0.5 ml Gentamicin solution (10mg/ml). To this solution, 25 ml of heat inactivated A^{+} (or AB^{+}) human serum and 12.5 ml 10% AlbuMAX I were added and the solution was stored at RT and supplied with gas after every use. Fresh blood was collected from a human volunteer with the anti-coagulant ACD, mixed well and immediately diluted by an equal volume of ICM. Diluted blood was centrifuged and the mononuclear cells at the sample/medium interface were removed and the RBCs were diluted with an equal volume of CM and used for culture or stored at 4°C. To start the culture, the cryo-preserved RBCs infected with the GFP-expressing *P. falciparum* were rapidly thawed in 37°C water bath and immediately transferred to a 50 ml sterile tube. 0.2 volumes of sterile 12% NaCl were added to the tube followed by 9 volumes of sterile 1.6% NaCl dropwise. The solution was centrifuged, aspirated and 9 volumes of sterile 0.9% NaCl + 0.2% Dextrose were added and mixed well. Supernatant was aspirated after centrifugation and the pellet was re-suspended in 5 ml of 37°C CM and transferred to a T25 sterile culture flask. Finally, 0.2 ml of 50% RBCs was added and the flask was supplied with gas, incubated at 37°C. Culture was maintained under 10 nM final concentration of the drug WR99210. Parasitemia was estimated by Giemsa staining of blood smears.

Synchronization of Parasite Cultures: *P. falciparum* culture was synchronized by 2 methods; 5% Sorbitol and Percoll gradient [13-14] with some modifications. First, infected RBCs were spin down, resuspended in 1-2 ml CM and 10 volumes of 5% Sorbitol. The suspension was incubated at 37°C for 8 min., spin down and immediately Sorbitol solution was removed. The pellet was washed once with 10 ml of ICM, resuspended in CM and dispensed to a sterile flask. Second, the culture was synchronized in the same way once more 26 hours later. Second, parasites were let to grow for one cycle and then synchronized using Percoll L-Alanine method to collect schizonts. In this method, three Percoll-alanine solutions were prepared as follows: 90% Percoll-Alanine solution was prepared by dissolving 77.3 gm of L-Alanine in 225 ml Percoll and 25 ml of 10X RPMI medium 1640 by moderate stirring. 70% Percoll-Alanine solution was prepared by mixing 12.5 ml of 90 % Percoll-Alanine with 3.5 ml of 1X RPMI medium 1640. 40% Percoll-Alanine solution was prepared by mixing 7 ml of 90% Percoll-Alanine and 9 ml of 1X RPMI medium 1640. To prepare Percoll gradient, 3 ml of 90% Percoll solution were added slowly to a 14 ml conical tube followed by adding 2 ml of 70% Percoll solution slowly. Finally, 2 ml of 40% Percoll solution were added slowly to the top of 70% Percoll solution. Parasite cultures were transferred to 50 ml tubes and centrifuged at 400 g for 10 min at 37 °C. Supernatants were aspirated carefully and a suitable amount of complete medium was added to each tube to achieve 30-40% hematocrit. This

RBC solution was added slowly to the prepared percoll gradient and centrifuged at 1200G for 10 min at 37 °C. Mature schizonts were collected from the interface between the 40 and 70% Percoll layer. The collected parasites were placed into a 50 ml conical tube and an equal volume (or more) of complete medium was added slowly to them and centrifuged at 400G for 10 min at 37 °C. After aspirating the supernatant, the desired amount of media was added and subdivided into a suitable number of culture flasks containing fresh RBCs and maintained for growth. Parasite rupture and reinvasion of RBCs was monitored and the cultures were examined at short intervals.

Fluorescence Microscopy: Small aliquots of parasite culture were collected every 4 hours, washed with PBS. For nuclear staining, parasites were incubated in PBS containing 4', 6-diamidino-2-phenylindole (DAPI) for 5 min and mounted. Fluorescent parasites were carefully investigated and imaged using fluorescence microscope supplied with a digital camera. Images were processed by using Adobe Photoshop software.

RESULTS

Formation of the Parasitophorous Vacuoles

In the Asexual Blood Stages: In the asexual blood stages of the parasites, no fluorescence signal was detected in ring or trophozoite stages whereas early schizont stages showed a uniform green fluorescence throughout the cell and maximal expression at the rim of the parasite (Fig. 1, row A). This fluorescence pattern is consistent with localization of the Clag3.1-GFP chimeric protein within the parasite cytoplasm and the PV. With the growth of parasites (3 nuclei stage), the fluorescence signal was detected in several extensions bulging out from the parent parasitophorous vacuole (Fig. 1, row B). More developed schizonts showed larger number of these extensions (Fig. 1, row C). In mature schizonts, a segmented fluorescence pattern surrounding the individual merozoites was observed; consistent again with localization of the Clag3.1-GFP chimeric protein to the PV (Fig. 1, row D and E). At this stage, no extensions from the parasitophorous vacuole were seen.

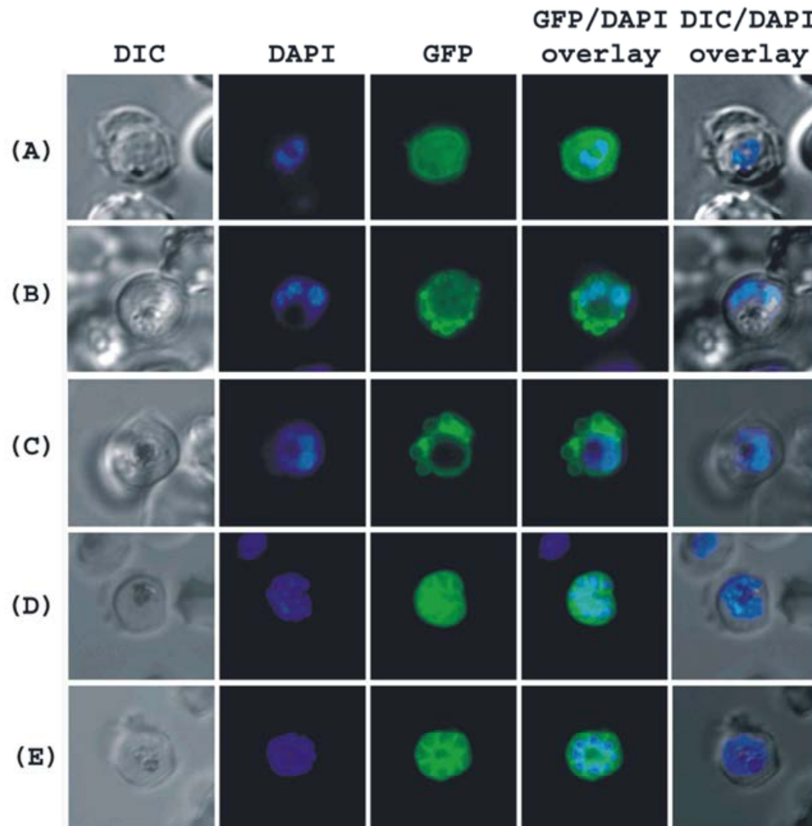

Fig. 1: Formation of the parasitophorous vacuole during the development of schizont. Roz A: early schizont stages. Row B and C: middle stage schiznts. Row D and E: late schizont stages.

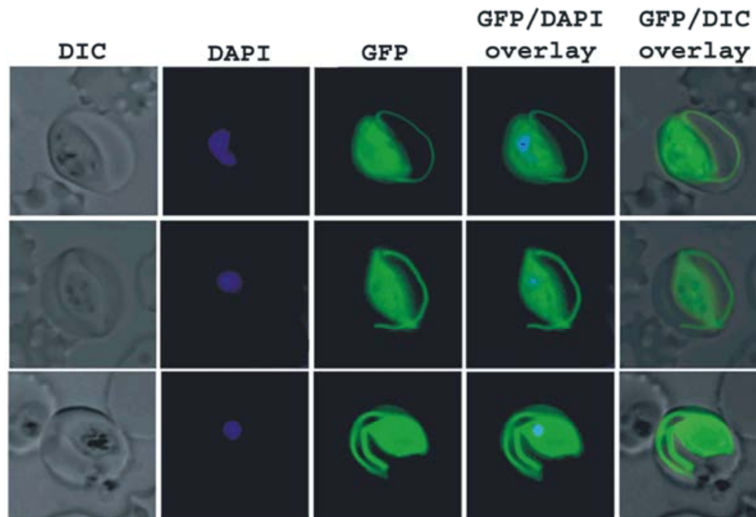

Fig. 2: Formation of the parasitophorous vacuole in game-tocytes.

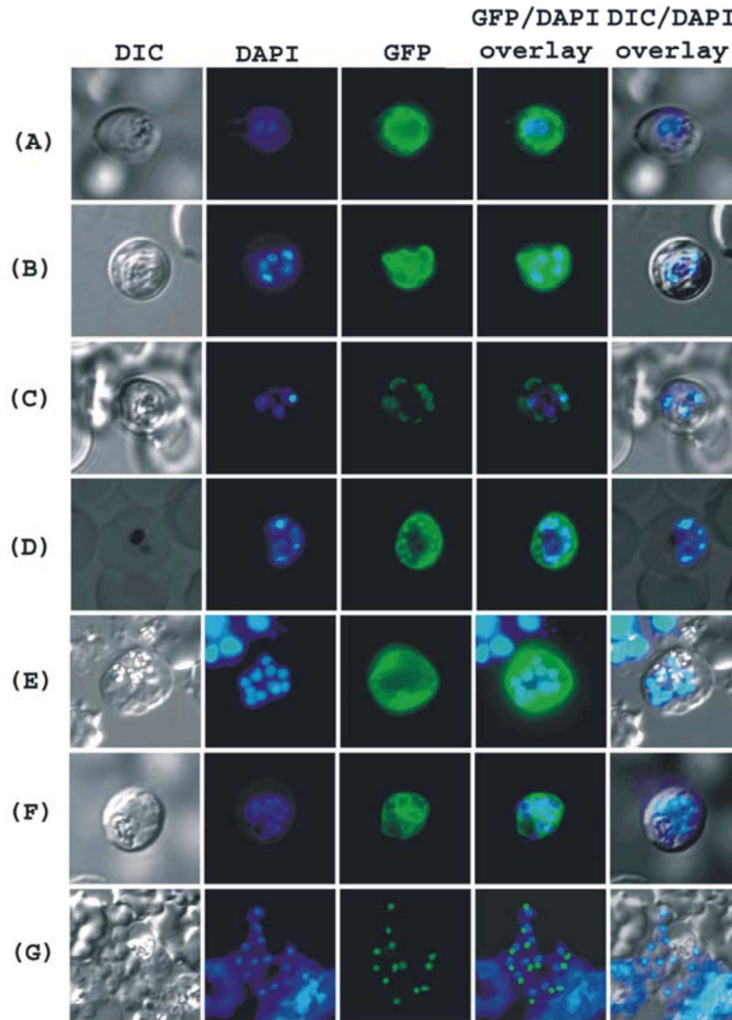

Fig. 3: Formation of rhoptry organelles during the development of schizont. Row A: early schizont. Rows B, C and D: middle stage schizonts. Row E and F: late schizont stages. Row G: ruptured schizont.

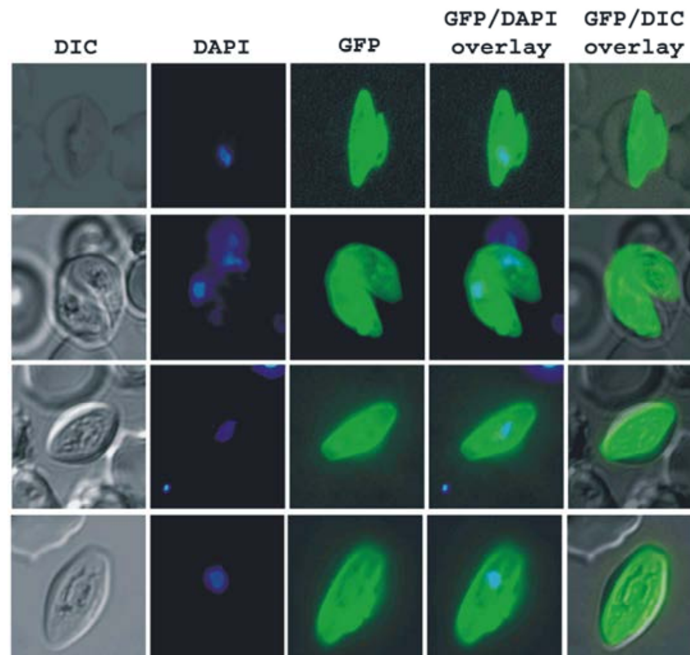

Fig. 4: Tracking rhoptry organelles in gametocytes

In the Sexual Blood Stages: In gametocytes, a uniform green fluorescence was also observed throughout the cell with a greater expression at the periphery of the gametocyte which may indicate the localization of the GFP chimeric protein within the gamete cytoplasm and the parasitophorous vacuole. In some cases, probably in more developed gametocytes, green fluorescent long tubular structures were observed. These tubular structures were seen single in some cases and in some others they were double and running parallel to each other. In all cases, these tubular structures were seen extending from the mother parasitophorous vacuoles and curving under the erythrocyte membrane (Fig. 2).

Formation of Rhoptry Organelles

In the Asexual Blood Stages: The green fluorescence signal was detected in the parasites only after the commencement of nuclear division. At the early stages (2 nuclei stage) of schizont development, the green fluorescence signal was localized in dense crescent-like areas at the periphery of the schizonts (Fig. 3, row A). The number of these areas was all the time more than the number of nuclei. In some cases, these areas surrounded the nuclear lobes (Fig. 3, row B and C). With the progress of schizont development (more than 4 nuclei), dense green fluorescent vesicles were seen distributed randomly at the periphery of the schizonts (Fig. 3, row D and E). Once the cytoplasmic divisions were complete, the individual merozoites were seen each surrounded by a segmented fluorescence pattern, which may reflect the formation of individual parasitophorous vacuoles. Every schizont was seen with its nucleus with apically located green fluorescent rhoptries (Fig. 3, row F). Later, mature schizonts ruptured and released their merozoites apically punctuated with rhoptry organelles (Fig. 3, row G).

In the Sexual Blood Stages: In most cases, a single green fluorescent signal was observed at sub terminal position in the gametocyte (Fig. 4). Occasionally, two signals were seen in different locations in the gametocyte cytoplasm, one of them was sub terminal and the other was centrally located around the nucleus. Remarkably, these fluorescent signals were observed at the early stages of gametocyte development. No such signals could be observed at the late gametocyte stages.

DISCUSSION

Formation of the Parasitophorous Vacuoles: Maturation of the parasite during the ring-trophozoite stage is characterized by the development of important PVM extensions into the host cell cytosol. These membranes comprise two types of projections [7, 15]. The first type of extensions is flattened plate-like structures with a translucent lumen and an electron dense coat, also called the Maurer's clefts. Although described as gradually relocating from near the PV to closer to the RBC membrane, these structures were never shown to connect

with the erythrocyte membrane. In the current study, no green fluorescent structures similar to Maurer's clefts were observed and this indicates the lack of a direct connection between the PV and Maurer's clefts. The second type of parasite membrane structures found within the RBC cytosol, are short and large tubes, initially continuous with the PVM and which terminate in vesicle-like enlargements. Altogether, these membranous tubes, distinguished by specific protein domains and extending the PVM into the cytosol, form a continuous network designed as the 'Tubulovesicular Network' (TVN) [7, 15]. The GFP chimera used in the current study detected several extensions bulging from the parent parasitophorous vacuole during the growth of schizont stages. Number of these extensions increased with the development of schizonts. On the contrast of a previous report [16] that free vesicular elements were described in the red blood cell cytoplasm, no such free vesicles could be observed during this study. To our knowledge, the presence of such vesicles was not confirmed yet.

TVN plays an important role in the parasite biology. Accumulating data showed that the TVN would be required for the uptake of nutrients from the host cell. At least a subset of raft components initially present in the red blood cell membrane were shown to be internalized into the newly formed vacuole and/or TVN [5]. Furthermore, low molecular mass solutes were detected in the TVN lumen, suggesting their import into the PVM from the extracellular medium [17]. Erythrocytes are known to be devoid of the endocytic and biosynthetic secretory machinery present in other cell types. Therefore, it has been hypothesized that *Plasmodium* would export the secretory machinery for protein traffic outside its own plasma membrane. A sphingomyelin synthase activity was detected at the TVN, whereas ERD2, a specific marker of the cis-Golgi in mammalian cells, was localized inside the parasite, indicating a bipartite functional repartition of Golgi activities in *Plasmodium*, with a cis-Golgi compartment localized inside the parasite. The TVN is considered to be functionally equivalent to Mammalian TGN [18]. The *Plasmodium* PVM and TVN have been considered as intermediate compartments in the trafficking to the Maurer's clefts [19, 20] which in turn constitute a Golgi-like sorting compartment of the parasite secretory machinery for proteins destined to the host cell surface knob structures [19, 21-23].

Targeting of parasite proteins to the PV is now well documented. All proteins transported into the RBC that have been analyzed to date, traverse the PV. PV sub-compartments might allow the discrimination between PV-resident and forward destined proteins. Importantly, a vacuolar export signal motif (PEXEL) was identified in 250 [24] or 400 [22] parasite proteins. This export signal exists in both soluble and membrane proteins. It is important to clarify that the presence of Clag3.1-GFP chimeric protein in the PV in the current study is more likely to be a kind of default residence rather than a directed targeting due to the absence of a PEXEL motif in this chimeric protein.

Sexual commitment occurs at the trophozoite stage during the previous asexual life cycle prior to DNA synthesis. Following the decision to switch to sexual stage development all the daughter merozoites become either all male or female gametocytes [25, 26]. In contrast, the process of gametocytogenesis occurs after a sexually committed merozoite invades a new erythrocyte. Up to our knowledge, there is no report on the presence and the shape of TVN in the sexual stages of the Apicomplexan parasites. The current study clearly shows that TVN in gametocytes is very well developed and extend more peripherally toward the surface of the host cell. This may refer to the possibility of direct contact of the gamete with the surrounding environment and consequently the high sensitivity of gametocytes to the changes in the host or the high need of the gametocyte to a continuous supply of nutrients. In fact, the changes in the culture medium resulting from parasite growth increase the rate of gametocyte formation in malaria cultures [27, 28]. *P. falciparum* gametocytes switch from one environment to another and this must involve the readiness of parasite to considerable metabolic changes considering the major differences between the two microenvironments [29].

Once sucked by the mosquito, the rich milieu of the blood is modified by mosquito factors such as serine proteases and chymotrypsins [30] and there is a drop in temperature and pH and, importantly, the parasite becomes extracellular from then on [29]. The well developed TVN detected in this study in gametocytes may implement the possibility of the need for a fast influx of mosquito factors to the inside of gametocyte through the TVN just before the release of gametes.

Formation of Rhoptry Organelles: Rhoptries are the largest of the *Plasmodium* secretory organelles and contain more than 20 proteins, many of which are unusual and have no recognizable orthologues, even in the closely related apicomplexan parasite *Toxoplasma gondii* [31]. It is known that rhoptry biogenesis is not co-ordinate with nuclear division. According to Jaikaria *et al.* [32], at the four-nucleus stage a vesicle could be isolated by sucrose gradient fractionation and this could represent an intermediate or pre-rhoptry compartment. At the 8-nucleus stage, a vesicle of low density was isolated.

At the 16-nucleus stage, vesicles having a significantly greater density similar to that of the mature organelle could be isolated. The study suggested that the rhoptry proteins first accumulate in a low density vesicle and that protein assembly into this compartment is staggered.

Rhoptry biogenesis occurs by sequential fusion of Golgi-derived vesicles which deliver protein cargo into the rhoptry lumen [33, 34]. Rhoptry proteins pass through the endoplasmic reticulum (ER) and the Golgi [35, 36]. The current study showed that at the early schizont stages the Clag3.1-GFP chimera was localized in peripheral crescent-like areas which surrounded the nuclear lobes. It is not clear yet whether the accumulation of proteins in specific regions is for a functional reason or just as a default before the formation of rhoptry organelles. There is a possibility that these accumulations could be to the inner side of PV (i.e. in the parasite cytoplasm); a possibility that need to be confirmed by colocalization of this GFP chimera with a PV authentic protein. Some of our presented images show these crescent areas in a wavy appearance that may support this possibility. The randomly distributed vesicles seen at the periphery of the schizonts in this study may represent the intermediate or pre-rhoptry compartments mentioned above by Jaikaria *et al.* [32]. These vesicles were located apically in every mature individual merozoite and have been previously shown by Ghoneim *et al.* [11] to represent the mature rhoptries by the use of colocalization studies with the authentic rhoptry protein RhopH2.

The observed green fluorescent signal in a subterminal and a central position in the cytoplasm of gametocytes seems to be a localized signal. However, rhoptry organelles have never yet been reported in the studies carried out by Talman *et al.* [29]; Aikawa *et al.* [37]; Sinden [38]; and Silva *et al.* [39] which described the ultrastructure of gametocytes in *Plasmodium* or any other parasite. One possibility is that these signals could be prerhoptry vesicles formed before the cell is destined to be a gametocyte. Another possibility is that these signals could be a non specific accumulation of the chimeric protein in some cytoplasmic organelles. In fact, non-specific expression timing leads to the accumulation of the chimeric proteins in the cytosol and/or cytosolic structures of the parasite [40, 41].

CONCLUSION

Using the green fluorescent protein as a biomarker, we tracked the formation of rhoptry organelles and the parasitophorous vacuoles in live *Plasmodium* parasites. Our data recorded new details during the formation of these vital organelles. This augments our information about the biology of this pathogen and could help to find new ways to intervene with the invasion ability of this lethal pathogen.

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