Origin, composition, organization and function of the inner membrane complex of Plasmodium falciparum gametocytes

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Abbreviations: GAP, Glideosome-Associated Protein; IMC, inner membrane complex; MyoA, myosin-A; MTIP, myosin-A tail domain interacting protein; PfERC, P. falciparum ER calcium binding protein; SIM, Structured Illumination Microscopy
Summary
The most virulent of the human malaria parasites, *Plasmodium falciparum*, undergoes a remarkable morphological transformation as it prepares itself for sexual reproduction and transmission via mosquitoes. Indeed *P. falciparum* is named for the unique falciform or crescent shape of the mature sexual stages. Once the metamorphosis is completed the mature gametocyte releases from sequestration sites and enters the circulation making it accessible to feeding mosquitoes. Early ultrastructural studies showed that gametocyte elongation is driven by the assembly of a system of flattened cisternal membrane compartments underneath the parasite plasma membrane and a supporting network of microtubules. Here we describe the molecular composition and origin of the sub-pellicular membrane complex, and show that it is analogous to the inner membrane complex, an organelle with structural and motor functions that is well conserved across the apicomplexa. We identify novel cross-linking elements that may help stabilize the inner membrane complex during gametocyte development. We show that changes in gametocyte morphology are associated with an increase in cellular deformability and postulate that this enables the gametocytes to circulate in the blood stream without being detected and removed by the mechanical filtering mechanisms in the host's spleen.

Introduction
Malaria affects more than 240 million people annually and causes about 781 thousand deaths (WHO, 2010). The apicomplexan parasite *Plasmodium falciparum* is responsible for the most deadly form of the disease in humans. The parasite has a complex lifecycle involving asexual and sexual reproduction within a human host and an *Anopheles* mosquito vector. To prepare itself for life in these different intracellular niches the parasite undergoes a remarkable series of morphological transformations, changing its own shape and remodeling its host cell environment. The link between asexual multiplication in the red blood cells (RBCs) of the human host and sexual reproduction in the midgut of the mosquito is the formation of a specialized sexual blood stage parasite called the gametocyte. Late stage *P. falciparum* gametocytes are characterized by their unique crescent or falciform shape. Development of these specialized cells takes at least 7 days and can be divided into 5 distinct stages (Baton and Ranford-Cartwright, 2005; Carter and Miller, 1979; Dixon et al., 2008; Hawking et al., 1971). Early gametocytes (stage I and Ila) are morphologically indistinguishable from early asexual parasites. Changes in cellular architecture are first observed during late stage II to stage III of development, leading to the characteristic crescent shape in stage IV, with the gametocyte ends become more rounded in stage V parasites (Dixon et al., 2008; Sinden, 1982; Sinden et al., 1978).
Like mature asexual stage parasites, stage I–IV gametocytes sequester away from the peripheral circulation (Day et al., 1998; Rogers et al., 1996), thereby avoiding passage through and potential clearance by the spleen. It has been suggested that stage I-II gametocytes adhere to CD36, before undergoing a switch in stage III / IV that permits sequestration in the bone marrow, while the mature stage V gametocytes lose the ability to cytoadhere (Rogers et al., 2000). Indeed stage V gametocytes reappear in the peripheral circulation and this is the only stage that can complete sexual development upon ingestion by a mosquito. Gametocyte maturation thus represents a “bottle neck” in parasite development; inhibition of this process would ablate disease transmission. Despite the importance of this stage in efforts to eradicate malaria, relatively little is known of the mechanisms controlling its shape, form and function.

Coincident with the adoption of the unique crescent shape of the late stage gametocyte is the appearance of a tri-laminar membrane structure known as the sub-pellicular membrane complex. Early electron microscopy studies on mammalian malarial parasites identified this structure as a double-membraned cisternal compartment underlying the parasite plasma membrane (Sinden and Smalley, 1979). In this work we describe some of the molecular components of the sub-pellicular complex, and show that it is closely related to the inner membrane complex (IMC) of the invasive stages of the parasite.

In the motile (zoite) stages of plasmodium and other apicomplexa the IMC is connected to an actin-myosin motor that drives cellular invasion through a process called gliding motility. The glideosome proteins include myosin-A (MyoA), myosin-A tail domain interacting protein (MTIP) (Bergman et al., 2003; Herm-Gotz et al., 2002) and the Glideosome-Associated Proteins 45 and 50 (GAP45/50) (Gaskins et al., 2004). PfGAP50 is an integral membrane protein that anchors pre-complexed GAP45-MTIP-MyoA (Johnson et al., 2007). Host cell invasion is thought to require binding of the actin-myosin complex to adhesive proteins, such as the merozoite thrombospondin-related adhesive protein (mTRAP). mTRAP is embedded in the parasite plasma membrane and binds to receptors on the host cell, thereby providing the force that drives invasion (Baum et al., 2006a). Underlying the IMC is a microtubule scaffold that forms a major structural element of the invasive stages of apicomplexan parasites (Kudryashev et al., 2010; Sinden, 1982).

We show that the sub-pellicular membranes of gametocytes have the same core components as in the invasive stages and that these components are in complex. Secondly, we provide evidence that a coordinated recruitment of the IMC to the parasite periphery and assembly of a network of microtubules drives elongation of the \textit{P. falciparum} gametocyte, confirming at a molecular level
suggestions from early electron microscopy studies (Sinden, 1982). This shape change is associated with an increase in cellular deformability and may explain how late stage gametocytes can survive circulation through the host’s blood stream.

Results

**IMC proteins are present in complex at the periphery of mature P. falciparum gametocytes**

The late stage *P. falciparum* gametocyte is characterized by its unique crescent shape. Coincident with the adoption of this shape is the appearance of a tri-laminar structure known as the sub-pellicular membrane complex, consisting of a cisternal compartment underlying the parasite plasma membrane (Sinden and Smalley, 1979). We performed high-resolution serial section electron tomography of this region in a mature stage gametocyte. Figure 1A shows (i) a virtual section from the tomogram and (ii) a rendered model of the membrane complex. The RBC membrane (red), parasitophorous vacuole membrane (PVM, blue), parasite plasma membrane (PPM, yellow), the sub-pellicular membranes (green) and a layer of sub-pellicular microtubules (MT, gray) are evident.

We were struck by the ultrastructural similarities between this sub-pellicular complex (Figure 1A) and the IMC of merozoites (Figure 1B). In the merozoite the IMC is a cisternal compartment that is flattened against the parasite plasma membrane (Figure 1Bi). It is supported by two or three longitudinally running microtubules that extend along one side of the merozoite (Figure 1Bii, arrow), as reported previously (Bannister et al., 2000).

While previous studies have described the sub-pellicular membrane complex of the *P. falciparum* gametocyte at the ultrastructural level, there are few reports of the composition or origin of this organelle. In an effort to determine whether the gametocyte membrane structure is indeed related to the IMC, we performed fixed cell immunofluorescence microscopy using antibodies recognizing known components of the merozoite IMC. Figure 2A-D shows the presence of PfGAP50, PfGAP45, MTIP and MyoA at the periphery of stage IV gametocytes, consistent with a location in the sub-pellicular membrane complex. At this level of resolution the profile is similar to that of the PVM protein, Pfs16, which has been shown to be gametocyte-specific (Baker et al., 1995). Protein solubility and expression profiling by western blotting of gametocytes and schizont stage parasites confirmed the presence of the IMC components (Figure S1, and below).

We have previously generated transfectants expressing a GFP chimera of PfGAP50 as a tool to assess IMC genesis and re-organization during the development of merozoites (Yeoman et al., 2011). We transfected the *PfGAP50-GFP* gene construct into a high gametocyte producing clone of 3D7 and
found that the PfGAP50-GFP chimera is correctly expressed and present at the periphery of mature stage gametocytes, co-locating with endogenous PfGAP45 (Figure 3A). To determine whether components of the IMC are in complex we performed immunoprecipitation experiments. Stage III-IV gametocytes expressing the PfGAP50-GFP chimera were magnet-enriched, then solubilized using RIPA detergent. The cleared lysate was incubated with rabbit anti-PfGAP45 antibodies, and immunoprecipitated using Protein A/G Sepharose. Precipitated samples were separated by SDS-PAGE and transferred for Western blotting. Blots were probed with anti-mouse GFP, PfGAP50 or MTIP. Full length PfGAP50-GFP (64 kDa) was detected in the precipitated pellet of the transgenic sample, by both the anti-GFP and anti-PfGAP50 reagents, but not in the 3D7 parent line (Figure 3B). MTIP (28 kDa) and endogenous PfGAP50 (42 kDa) were detected in the precipitated pellets of both the transfectant and wild type samples (Figure 3B). This demonstrates that both PfGAP50-GFP and endogenous PfGAP50 are in complex with endogenous PfGAP45 and MTIP. These studies validate the use of PfGAP50-GFP as a marker of the gametocyte IMC.

Western blotting analyses revealed the presence of PfGAP45, PfGAP50 and MTIP from stage II to stage V gametocytes (Figure S1B) and immunofluorescence microscopy confirmed that these proteins were present at the gametocyte periphery (Figure S2A-D). Moreover the solubility profiles for these proteins are similar in gametocytes and merozoites (Figure S1). These data in conjunction with the immunoprecipitation data show that the gametocyte sub-pellicular membrane complex is very closely related to the merozoite IMC and we suggest that it be renamed the gametocyte IMC.

**PfGAP50-GFP is present in the endoplasmic reticulum (ER) of early stage gametocytes prior to recruitment to the parasite periphery**

PfGAP50 is recruited from the ER to the nascent IMC during asexual schizogony (Yeoman et al., 2011). To determine the origin and reorganization of proteins during the formation of the gametocyte IMC we examined the location of PfGAP50-GFP in live gametocytes that were co-labeled with the membrane probe, BODIPY-ceramide. During the initial stages of elongation (stage III) when the gametocyte first becomes morphologically distinguishable, PfGAP50 is associated with a reticular structure in the parasite as well as along one side of the elongating parasite (Figure 4Ai). Fixed cell immunofluorescence microscopy confirmed that PfGAP50-GFP labels internal structures that are also recognized by an antibody against the ER resident protein, PfERC (ER calcium binding protein) (Figure 3C). By contrast PfERC is absent from the peripheral location to which PfGAP50-GFP is recruited as the gametocyte elongates. 3D-Structured Illumination Microscopy (3D-SIM) can provide
enhanced resolution of cellular structures (Schermelleh et al., 2008). Using 3D-SIM we found that the PfGAP50-GFP-labelled IMC remains closely associated with the intracellular reticular structure (Figure 3D,E, arrows). A rendered model of the GAP50-GFP fluorescence illustrates the points of associations (Figure 3F, arrows), as do translational and rotation views of the fluorescence micrographs (Movies S1,2). This is consistent with an early ultrastructural study (Sinden et al., 1978) which reported connectivity between the ER and the sub-pellicular membranes.

Stage III gametocytes undergo a dramatic cellular rearrangement adopting a “hat-like” appearance. During this stage the PfGAP50-GFP is concentrated in the flattened rim region of the “hat” with additional looping structures around the top of the cell (Figure 4Ai,ii arrows; see Movie S2). As the IMC develops further it adopts a cupped shape along the foot, in addition to the looping structures. Electron microscopy confirms the presence of the IMC along the flattened surface and around the “pinching” ends of the gametocyte (Figure 4Bi,ii). Figure 4B(i) depicts a longitudinal slice through a region of the sub-pellicular microtubule layer with the IMC visible at the periphery. Some of the microtubules that pass through the plane of the section are indicated with the arrows. Figure 4B(ii) shows a cross section in which the microtubules are viewed end on and the IMC is evident as a layer outside the microtubule "basket".

In stage IV of the parasite’s development the characteristic pointed ends of the *P. falciparum* gametocyte become apparent. By this stage the PfGAP50-GFP extends further around the parasite, the surface of which is delineated by BODIPY-ceramide labeling (Figure 4Aiii; see Movie S2 for a 3D rotation). 3D-SIM imaging of PfGAP50-GFP reveals a periodic banding pattern running perpendicular to the direction of gametocyte extension (Figure 5A, arrows). The average width of the PfGAP50-GFP fluorescence bands is 460 ± 40 nm with the interleaved regions of low fluorescence being 120 ± 40 nm in width (Figure 5A, Movie S3).

Cryo electron tomography allows 3D visualization of specimens after plunge-freezing in cryogenic liquids to preserve membrane structures (Cyrklaff et al., 2007; Kudryashev et al., 2010). Tomographic analysis of a stage IV gametocyte permits visualization of virtual sections near the surface of the cell without the need for physical sectioning. Figure 5Bi highlights the microtubule network running underneath the cell surface and the locations of the IMC and parasite membranes within the cell. Figure 5Bii (physically located 100 nm above the section in Figure 5Bi) shows regions of membrane (approximately 400 nm in width) interleaved with connecting bands, with a width of about 100 nm, layered across the underlying microtubules. Higher magnification view of the microtubules (Figure 5C) highlight the spacing and arrangement of the microtubule network within the mature stage parasite; the
microtubules (25 nm diameter) are spaced at intervals of approximately 10 nm. The spacing, shape and orientation of the flattened membrane structures are reminiscent of the PfGAP50-GFP-labeled features (Figure 5A,S3, Movie 5a,b). The intervening bands may represent areas of deposition of proteins that stabilize the IMC and the underlying microtubule network.

In stage V the ends of the gametocytes become rounded (Figure 4Aiv, 5Aiii). This is likely due to the dismantling of the supporting microtubule network as described below and in a previous study (Sinden et al., 1978). PfGAP50-GFP persists at the periphery of the parasite still co-locating with BODIPY-ceramide (Figure 4Aiv). 3D-SIM reveals the maintenance of the IMC around the cell's periphery, and the persistence of the stripes (Figure 5Aiii, arrows). The gametocyte presented in Movie S3 shows the transition from stage IV to stage V with one rounded and one pointed end. In this rotation the stripes are clearly observed and there is evidence for some remnant PfGAP50-GFP fluorescence around the parasite nucleus.

**Temporal and spatial map of gametocyte IMC and microtubules at different stages of gametocyte development**

We investigated the assembly of microtubules during gametocyte development. The microtubule-labeling reagent, Tubulin Tracker™ (Invitrogen) starts to accumulate in a peripheral region of a stage II gametocyte (Figure 6Ai, Movie S4) before becoming concentrated along one edge of the stage III gametocyte (Figure 6Aii, Movie S4). Extension and elaboration of the microtubule network is observed in stage IV gametocytes (Figure 6Aiii, Movie S4), adopting a pattern analogous to that of the peripheral population of PfGAP50-GFP (Figure 4Aii). No microtubule filament bundles are observed in the stage V gametocyte, consistent with the disassembly of the network in mature gametocytes.

We have used the enhanced resolution of 3D-SIM to investigate the locations of the microtubules and IMC during gametocyte elongation. Anti-β-tubulin labels microtubules within the “foot” of the developing gametocyte (Figure 6B, white arrows) in addition to a diffuse cytoplasmic staining. β-tubulin in the “foot” region is co-located with PfGAP50-GFP (Figure 6B). The distribution of the microtubules and the IMC can best be appreciated in Movies S5a,b, which shows a translational through and a rotation of the same parasite. PfGAP50-GFP and β-tubulin are also co-located in the loop around the top of the gametocyte (Figure 6Bii; Movies S5a,b). Some PfGAP50-GFP-labelled regions around the edge of the foot do not have associated microtubules (Figure S3; Movies S5a,b). Electron micrographs of equivalent stages show areas where the IMC has started to expand but there are not yet underlying microtubules (Figure 4Bii, red arrows). By stage IV there is a complete network
of microtubules underlying the peripherally located IMC and forming a similar pattern to that for PfGAP50. This characteristic shape and the distribution of the microtubules can be best appreciated in the 3D-SIM reconstruction shown in Movie S6.

During stage III we see a marked difference in the locations of PfGAP45 compared with PfGAP50-GFP and β-tubulin. This protein is restricted to the foot and is not co-located with the ER-associated population of PfGAP50-GFP (Figure 6B,C,S2B).

**Gametocyte elongation is not driven by an actin-myosin motor**

Gametocytes do not display gliding motility and it is unlikely that the IMC and associated MyoA participate in a motor complex analogous to that operating in the zoite stages of *P. falciparum*. To test for a potential role of actin we examined the effect of cytochalasin D on gametocyte morphology. Previous work has shown that merozoites treated with cytochalasin D (in the 0.1 to 2 μM range) are rendered immobile and unable to invade erythrocytes (Miller et al., 1979; Srinivasan et al., 2011). By contrast, we found that maintenance of gametocytes over a period of 6 days in the presence of cytochalasin D (at concentrations up to 10 μM) did not prevent the morphological changes that accompany development to stage IV (data not shown). This indicates that while MyoA interactions may play an important structural role, morphological changes are not driven by an actin-myosin motor.

**Gametocyte shape change is associated with altered cellular deformability**

In an effort to determine a functional role for the remarkable shape changes of gametocytes we used ektocytometry to measure cellular deformability at different stages of development (Figure 7). The elongation index (EI) of synchronous enriched gametocytes was measured at sheer stresses from 0-20 Pa. Asexual trophozoite stage parasites show a significant decrease in ability to elongate under flow (EI = 0.13) compared to uninfected RBCs (EI = 0.34) at the physiologically relevant sheer stress of 3 Pa (Figure 7A). Stage III gametocytes showed a similarly low deformability (EI = 0.12) (Figure 7A). By contrast there was an increase in the elongation index upon transition to stage IV of gametocyte development (EI = 0.16); this value is significantly higher than for stage III (P = 0.0001). A further increase in the elongation index was observed upon transition to stage V (EI = 0.18) (Figure 7A).

We made a rough quantitation of the change in the shape at different stages of development by monitoring length and width in DIC images of live gametocytes. Mature trophozoite stage parasites display a roughly circular profile with a mean length of 4.31 ± 0.05 μm and mean width of 4.0 ± 0.2 μm) (Figure 7B,C). The mean length of the gametocytes increases significantly from stage III (6.6 ±
0.4 μm) to IV (10.9 ± 0.4 μm) (P = 0.0001) with a slight relaxation upon transition to stage V (9.1 ± 0.4 μm) (P = 0.005) (Figure 7B,C). The width of the stage III gametocyte (3.0 ± 0.7 μm) is significantly less (P = 0.0001) than that of the asexual stage parasite, with a further significant decrease in the stage IV gametocyte (2.6 ± 0.1 μm, P = 0.006), before remaining stable for the remainder of its development (stage V; 2.6 ± 0.2 μm) (Figure 7B,C). The length to width ratio increases from 1.1 (trophozoite) to 2.2 (stage III) to 4.3 (stage IV), and then decreases to 3.5 (stage V).

Discussion

In 1880, Alphonse Laveran observed gametocytes in the blood smear of an Algerian malaria patient and remarked upon the crescent shape of the parasite (Laveran, 1880). Over a century later we are still battling to understand the molecular basis for this unusual shape and its role in \textit{P. falciparum} transmission and virulence. Early ultrastructural analyses revealed that the gross morphological restructuring of the gametocyte is coincident with the appearance of a tri-laminar membrane structure subtended by a layer of structural microtubules (Aikawa and Beaudoin, 1969; Sinden, 1982; Sinden et al., 1978). Comprising a double membrane-bound compartment underneath the parasite plasma membrane, this structure was named the sub-pellicular membrane complex (Sinden and Smalley, 1979). Indeed the presence of alveolar sacks subtending the plasma membrane is a unifying morphological feature of apicomplexa, dinoflagellates and ciliates. Different organism groups have adapted this membrane structure for different cellular functions, with roles ranging from shape maintenance (free-living protozoa), to protective armor (dinoflagellates), to calcium stores (ciliates). In the motile stages of apicomplexan parasites, the sub-pellicular membrane is termed the IMC, and plays a vital role as the scaffold for the motor complex that drives parasite motility (Beck et al., 2010; Frenal et al., 2010; Gaskins et al., 2004).

Our work confirms previous studies showing that the gametocyte sub-pellicular membrane complex has ultrastructural similarity to the IMC of the motile stages of \textit{P. falciparum} (Bannister et al., 2000; Sinden and Smalley, 1979), however the molecular similarity between these structures has not previously been investigated. In this work we show that key proteins of the merozoite glideosome complex, PfGAP50, PfGAP45, MyoA and MTIP, are present in the gametocyte sub-pellicular membrane complex. We also show that exogenous PfGAP50-GFP forms a complex with endogenous PfGAP45, PfGAP50 and MTIP. These data indicate that the gametocyte membrane complex is closely related to the IMC of the motile stages of \textit{P. falciparum}.
An interesting question is: what roles do the IMC and the associated glideosome proteins play in gametocyte assembly. Gametocytes do not display gliding motility, suggesting that the MyoA does not actively participate in an actin-myosin motor complex. Furthermore we showed that treatment with cytochalasin D did not prevent gametocyte shape change. This indicates that the MyoA interaction with glideosome proteins likely plays a structural role rather than driving elongation per se. It seems likely that the microtubular cytoskeleton is attached to (and organized by) the IMC which in turn is linked to the PPM via glideosome proteins. Thus our data confirm and support the original suggestion (Sinden, 1982) that gametocyte elongation is driven by the assembly of microtubules underneath the IMC.

The shape adopted by *P. falciparum* gametocytes is reminiscent of the elongated sporozoite and ookinete. Indeed the morphological changes that accompany maturation from stage II to stage IV of gametocytogenesis resemble (in reverse) the shape changes that accompany the transformation of the elongated sporozoites into liver stage trophozoites (Jayabalasingham et al., 2010) or ookinetes into oocysts (Carter et al., 2007). Taken together these data suggest that the IMC plays an important structural role in each of the cellular remodeling events that drive parasite elongation. This is consistent with recent work showing that specific IMC proteins can play roles in both motility and cell morphology (Tremp and Dessens, 2011).

Recent work from our laboratory indicates that the merozoite IMC is formed by the redistribution of a sub-compartment of the ER to nascent apical caps (Yeoman et al., 2011). Similarly we show here that PfGAP50 is located in the ER in early stage gametocytes and that part of this population is recruited to the periphery to form the IMC. This is in agreement with early ultrastructural study of *P. falciparum* (Sinden et al., 1978) and with studies from other alveolates (Gould et al., 2008). Recruitment of PfGAP50 to the gametocyte periphery appears to be coordinated with the laying down of microtubules. Because the microtubule-supported IMC initially develops along one side of the gametocyte, stage III gametocytes have a "hat-like" appearance. The IMC and the microtubule network then expand around the periphery resulting in a cupped leaf shape. Our electron and fluorescence microscopy data suggest that the IMC extends first, followed by microtubule deposition, such that the leading edges of the nascent IMC are outside the supporting layer of microtubules. Other IMC proteins, PfGAP45 and MTIP, appear to be recruited on to the nascent IMC. This process may be similar to the recruitment of pre-complexed GAP45-MTIP-MyoA onto membrane-embedded PfGAP50 during the formation of the *Toxoplasma* IMC (Gaskins et al., 2004).

3D-SIM imaging of the PfGAP50-GFP-labelled IMC revealed a remarkable architectural feature - bands of fluorescence of ~400 nm interrupted by ~100 nm regions of low fluorescence. Cryo electron
tomography revealed the ultrastructural analogue of these bands as flattened regions of membrane with intervening amorphous material. These structures are likely equivalent to the transverse “sutures” encircling the gametocyte that were identified in an early freeze fracture study (Meszoely et al., 1987). Similar patchworks of sub-pellicular membrane have been described in the IMC of the invasive stages of *P. falciparum*, as well as in *Toxoplasma* and other apicomplexa (Bannister et al., 2000; Raibaud et al., 2001). Another early transmission electron microscopy study identified protein “cross-links” running perpendicular to the microtubule network (Kaidoh et al., 1993). We suggest that the PfGAP50-GFP-labelled IMC “stripes” represent IMC cisternae laid down as rectangular plates, while the gaps represent proteinaceous material that holds the cisternae and the microtubules in place.

A concurrent large-scale analysis of IMC components in *P. falciparum* revealed a novel IMC protein that appears to be restricted to the genus *Plasmodium* (Mal13P1.228). In the early gametocyte this protein is located in thin stripes along the "foot", which expand and eventually encircle the mature stage gametocyte (M. Kono, T. Gilberger, personal communication). These structures may represent the regions of low fluorescence and proteinaceous cross-links identified in our 3D-SIM and cryo electron microscopy studies.

Consistent with previous ultrastructural studies of gametocytes (Sinden, 1982; Sinden et al., 1978), there is no morphologically identifiable apical polar ring in gametocytes to organize the peripheral microtubules (although an MTOC is formed very quickly after activation, (Aikawa, 1977)). This begs the question, how are the microtubules organized onto the well-ordered cytoskeletal basket. We suggest that the proteinaceous "sutures" between the IMC plates provide a means of stabilizing the microtubule network. Proteins resident in these "sutures" may function in the targeting of the nascent IMC to the periphery of the cell and the stabilization of the IMC and the sub-IMC microtubule network.

Analysis of DIC images revealed a significant increase in the length and a reduction in the width of the gametocyte during maturation when compared to asexual stage parasites. A more detailed analysis of gametocyte shape by cryo x-ray tomography gives similar results (Hanssen et al., 2011). The elongated form of *P. falciparum* gametocytes is unusual. Gametocytes from other human malaria species and gametocytes from most other species of *Plasmodium* have a more rounded morphology, although gametocytes of avian plasmodia are also elongate (Sinden et al., 1978). *P. falciparum* is also unusual amongst human malaria parasites in that late stage asexual parasites drastically remodel the RBC through the export and insertion of parasite-derived proteins into and under the host RBC membrane (Tilley et al., 2011). These modifications lead to a significant increase in cellular rigidity, which would make mature parasite-infected RBCs vulnerable to recognition and clearance within the spleen.
(Glenister et al., 2002; Safeukui et al., 2008). The parasite avoids these clearance mechanisms by cytoadhereing within the microvasculature of the host. Early stage *P. falciparum* gametocytes also sequester, however upon reaching sexual maturity they release from their sites of adhesion and re-enter the circulation to enable transmission to mosquitoes (Day et al., 1998; Hayward et al., 1999).

In an effort to determine the functional consequences of the gametocyte shape changes we investigated the ability of asexual trophozoites and stage III - V gametocytes to elongate in flow, a surrogate measure of cellular deformability (Groner et al., 1980; Hardeman et al., 1987). In agreement with previous reports we found that asexual trophozoite-infected RBCs are highly undeformable (Nash et al., 1989). This loss of deformability is likely due to a combination of membrane rigidification and the presence of the large rigid intracellular parasite (Glenister et al., 2002; Nash et al., 1989). We found that stage III gametocytes have similar deformability properties to trophozoite-infected RBCs, however upon transition to stage IV the gametocytes show a significant increase in the elongation index measured under flow conditions. This is likely due in part to the elongated shape. A further increase in the elongation index in stage V gametocytes may be due to the fact that the microtubule network is disassembled at this point leaving an already elongated cell that likely extends further under flow pressure.

These changes in rheological properties may help stage V gametocytes survive in the host’s circulation. Indeed stage V gametocytes may have rheological properties more akin to *P. vivax*-infected RBCs (Handayani et al., 2009; Suwanarusk et al., 2004). As its name suggests (*vivax* means lively), *P. vivax* is more amoeboid than *P. falciparum* and appears to avoid splenic clearance in spite of an inability to cytoadhere. Stage III-V gametocytes lack knobs and are thought to adhere with lower affinity than asexual stages and to prefer sites of reduced vascular flow (Rogers et al., 1996; Smalley et al., 1981). It is possible that another consequence of the elongated shape of *P. falciparum* gametocytes is enhanced adhesion to lower affinity receptors by permitting flattening of the cell, which may increase the area in contact with the capillary walls.

In conclusion our data show that the falciform shape of *P. falciparum* gametocytes is supported by an IMC with very similar composition to the merozoite IMC, linked to underlying microtubules. A previously unrecognized correlate of gametocyte elongation is an enhanced cellular deformability. We anticipate that disruption of the IMC and its associated microtubules would compromise the ability of *P. falciparum* to adopt the falciform shape; this may in turn affect its ability to survive in the circulation. This may provide a new avenue for interfering with this important human pathogen.
Materials and Methods

Parasite culture and gametocyte enrichment

Parasite-infected RBCs were cultured in RPMI-HEPES supplemented with 5% human serum and 0.25% AlbuMAX II as previously described (Foley et al., 1994). Laboratory strains of 3D7 cultured for long periods form gametocytes with low efficiency, therefore we have used a 3D7 isolate recovered from a patient (Lawrence et al., 2000) and cultured for only a limited time. A PfGAP50-GFP transgenic parasite was created by transfecting a high gametocyte producing clone of 3D7 with the pGLUX-PfGAP50-GFP construct as previously described (Yeoman et al., 2011).

Gametocytes were prepared using a modification of a published protocol (Fivelman et al., 2007). A culture of mainly ring stage parasites (6-8% parasitemia) was treated with 5% sorbitol (Lambros and Vanderberg, 1979), then separated using a Percoll density gradient (Knight and Sinden, 1982) to enrich the ring stage and remove any gametocytes. The parasites were cultured until they reached 8-10% trophozoites then sub-divided to 2% trophozoites (5% hematocrit). The culture was maintained for 10 days in the presence of 62.5 mM N-acetyl glucosamine to inhibit merozoite invasion and thus asexual replication (Hadley et al, 1986). Giemsa-stained slides were used to monitor stage progression. The medium was changed daily but no fresh erythrocytes were added. At the desired stages of development gametocytes were enriched by magnetic separation (Fivelman et al., 2007). For analysis of the effect of cytochalasin D, the drug (Sigma Aldrich) was added at concentrations from 0 - 10 μM at day one of gametocyte generation and supplemented daily into the culture media. The parasites were assessed by microscopy at different stages of development.

Microscopy and image analysis

Live infected RBCs were prepared for fluorescence microscopy as previously described (Dixon et al., 2011). Immunofluorescence was performed on acetone-fixed blood films (Spielmann et al., 2006) using the following primary antibodies in 3% BSA/PBS: mouse anti-GFP (1:500, Roche), rabbit anti-GFP (1:500), rabbit anti-PfGAP45 (1:500, (Baum, 2005)), rabbit anti-PfGAP50 (1:500, (Baum, 2005)), rabbit anti-Pfs16 (1:1000, (Baker et al., 1995)), mouse anti-Pfs16 (1:1000, (Dixon et al., 2009)), mouse anti-MTIP (1:500, (Green et al., 2006)), mouse anti-MyoA (Baum et al., 2006b) followed by anti-mouse or anti-rabbit IgG conjugated to Alexa Fluor 568, 647 or FITC. Slides were labeled with 1 μg/ml of DAPI prior to mounting.

BODIPY labeling of parasite-infected cells was performed by adding BODIPY-TR ceramide at a final concentration of 0.7μM to the parasite culture. Cells were incubated overnight under standard culture
conditions. Labeling with Tubulin Tracker™ Green was performed according to the manufactures instructions, using a final concentration of 250 nM and a 30min incubation at 37°C. Microscopy was performed using an Olympus IX81 widefield microscope, or Leica TCS-SP2 or Zeiss LSM 510/FCS confocal microscopes. Images were processed using NIH ImageJ version 1.42 (www.rsweb.nih.gov/ij). Samples were prepared and 3D-SIM (Microbial Imaging Facility, University of Technology, Sydney) performed as previously described (Yeoman et al., 2011). Analysis of the dimensions of at least 7 cells from DIC images was performed using Image J. Isosurface renderings of GAP50GFP expressing gametocytes were generated using IMOD software (Kremer et al., 1996; Mastronarde, 1997).

**Immunoprecipitation and solubility studies**

Gametocytes were harvested on days 3, 5, 7, 9 and 11 representing stages I to V. Pellets of magnet-purified gametocytes were lysed in 0.015% saponin on ice for 15 min. The saponin pellets were solubilized in SDS loading buffer and equal amounts (10 μg protein) were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting.

For solubility studies magnet-purified stage IV gametocytes were lysed in 0.015% saponin and the pellets were incubated for 15 min with 1% Triton 100 (Tx100) or RIPA (1% IGEPAL CA-630), 150 mM NaCl, 0.5% sodium deoxycholate on ice, or 0.1% sodium dodecyl sulphate (SDS), 50 mM TRIS, pH 7.4) or 2% SDS at room temperature and the soluble supernatant and insoluble pellet fractions collected. Equivalent amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting.

For immunoprecipitation studies purified parasites were lysed in RIPA buffer for 15 min on ice in the presence of protease inhibitor, centrifuged, and the supernatants collected. The precipitating agents were GFP-TRAP reagent (Chromotek) and protein A-Sepharose and anti-rabbit PfGAP45 (1:500) (Yeoman et al., 2011). The pellets were analyzed by SDS-PAGE and immunoblotting blotting.

Immunoblotting was performed as previously described (Dixon et al., 2011). Briefly all antibodies were prepared in 3.5% skim milk/PBS, and both primary and secondary incubations were performed for 1 hour. Three washes of PBS containing 0.1% Tween-20 were performed between each incubation. The following primary antibodies were used: mouse anti-GFP (1:500, Roche), rabbit anti-GFP (1:500), rabbit anti-PfGAP45 (1:500, (Baum, 2005)), rabbit anti-PfGAP50 (1:500, (Baum, 2005)), mouse anti-PfGAP50 (1:500, (Baum, 2005)), mouse anti-Pfs16 (1:1000, (Dixon et al., 2009)), mouse anti-MTIP (1:500, (Green et al., 2006)) and mouse MAb anti-AMA1 (MAb 1F9 1:500, (Coley et al., 2001)). Goat
anti-mouse or anti-rabbit IgG secondary antibodies conjugated to HRP were used. Electrochemiluminescence reagents and autoradiography were used to visualize the immunoblots.

**Cell Deformability**

Mature stage asexual (30-36 hour trophozoite) and stage III-V gametocytes were magnet purified from culture, washed in PBS, then reconstituted with uninfected RBCs to obtain a working parasitemia of 70% (Maier et al., 2008). The cell samples (5 μL) were mixed with 500 μl of polyvinylindone (PVP) solution at a viscosity of 25 mPa.s (Rheo Meditech Inc). The elongation index was measured in a RheoScan Ektocytometer, according to the manufacturer's instructions. Measurements were acquired over the 0-20 Pa range. Values shown are means plus or minus the standard error from a minimum of 3 individual measurements. The results shown are from a single experiment. The experiment was repeated showing comparable results (2 separate experiments).

**Electron Microscopy/ Tomography**

Transmission electron microscopy and tomography of asexual stages was performed as previously described (del Pilar Crespo et al., 2008; Hanssen et al., 2010), for merozoites the cells were post fixed with potassium ferricyanide reduced osmium tetroxide. Gametocytes from selected stages were fixed and embedded using a modification of a published protocol (Kass et al., 1971). That is cells were fixed in 5% glutaraldehyde in PBS, pH 7.3, overnight, then embedded in 3% agarose before undergoing post fixation of lipids in osmium tetroxide (1% in PBS) for 1 hour followed by progressive dehydration in ethanol. Samples were further dehydrated using progressive ethanol: acetone ratios of 2:1, 1:1 and 1:2, and finally immersed in acetone for 10 minutes. Acetone was gradually replaced with liquid epoxy resin, and samples embedded and stained as described above. For both asexual and gametocyte samples, 70 nm thick sections were observed at 120 keV using a JEOL-2010HC (Japan) transmission electron microscope (La Trobe University, EM Unit). For electron tomography, 200 or 300 nm sections were cut and stained and observed at 200 keV on a Tecnai G² F30 (FEI Company) transmission electron microscope (Bio21 Institute, Melbourne). Tilt series were collected every 2º between -70º and +70º and reconstructed to generate cell models as described previously (Abu Bakar et al., 2010; Yeoman et al., 2011). Tomograms were generated using the IMOD software (Kremer et al., 1996; Mastronarde, 1997). IMOD was also used to generate segmentation models where contours were assigned manually.
Cryo Electron Tomography
Gametocytes were transferred onto carbon-coated grids. After removal of excess liquid, grids were rapidly frozen by plunging into liquid ethane then transferred to liquid nitrogen. Grids were mounted on a Gatan cryo-holder and imaged using a Tecnai G² F30 (300 keV). Tilt series were collected every 2º between -70º and +70º. The total electron dose kept was 6000 e/\text{nm}^2.

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References


**Figure Legends**

**Figure 1.** The trilaminar complex of the gametocyte is closely related to the inner membrane complex of invasive stages of *P. falciparum*.

(A) Electron tomogram showing the membrane and microtubule organization in a stage IV gametocyte. (i) Selected virtual tomogram section (22 nm). (ii) Rendered model generated from four serial sections through the region shown in (i). The rendered model shows the RBC membrane (red), the PVM (blue), the PPM (yellow), the IMC (green) and microtubules (gray). Osmophillic bodies are represented in gold. (B) Stained thin section through a dividing schizont showing a nascent merozoite (i) and a selected virtual section (7 nm) from a tomogram of an individual merozoite (ii), showing a rhoptry (R), micronemes (m), the apicoplast (a), the nucleus (N), the PVM, PPM and IMC, and a microtubule (MT). Scale bars = 100 nm.

**Figure 2.** Components of the glideosome and IMC are present at the gametocyte periphery.

(A-D) Immunofluorescence microscopy of 3D7 parasites labeled with antibodies against PfGAP50, PfGAP45, MTIP or MyoA (green) and the gametocyte marker, Pfs16 (red). Scale bars = 5 μm.

**Figure 3.** PfGAP50-GFP is trafficked via the ER to the gametocyte IMC and is in complex with known IMC components.

(A) Immunofluorescence microscopy of PfGAP50-GFP expressing gametocytes labeled with antibodies against GFP (green) and PfGAP45 (red). Bright field (BF) images are at the right. Scale bars = 3 μm. (B) Proteins were detergent-solubilized from enriched, mature stage PfGAP50-GFP-transfected or parent 3D7 gametocytes. The extract was immunoprecipitated (IP) using rabbit anti-PfGAP45. Western blots of the pellet fractions were probed with mouse anti-GFP, anti-PfGAP50 and anti-MTIP. Molecular mass is shown in kDa. (C-E) Immunofluorescence (C) and 3D-SIM (D, E) of
PfGAP50-GFP transfectants labeled with anti-GFP (green) and anti-PfERC (red). DAPI fluorescence is shown at right. (C) An accumulation of GAP50-GFP fluorescence is observed along one side of the stage III parasite. (D) A projection of sections 10-15 (from a 20 section series), (E) individual sections, and a rendered model (F) of the 3D reconstruction, illustrate the association of the GAP50-GFP-labelled reticular and peripherally-located compartments. The single arrows indicate a region of close association between the ER and the IMC and the double arrows show a regions of IMC looping that appears to be continuous with the ER compartment.

Figure 4. The IMC is assembled under the gametocyte plasma membrane.
(A) Live cell confocal imaging of stage III to V gametocytes expressing PfGAP50-GFP (green) co-labeled with BODIPY-TR-ceramide (red). The white arrows indicate an accumulation of GAP50-GFP along the rim of the stage III parasite (i) and a loop around the top of the gametocyte (ii). 3D rotations of these cells are shown in Movie S2. Scale bars = 3 μm. (B) Electron micrographs of stage III gametocytes. (i) Longitudinal and (ii) cross sections showing the RBC membrane, PVM, PPM, and the double membrane of the IMC, above a row of microtubules (MT, arrowheads). The electron-lucent area likely represents a vacuole within the gametocyte cytoplasm. The red arrows mark an area where the IMC has formed but no microtubules are present. Scale bars = 200 nm. See also Figure S2.

Figure 5. The gametocyte IMC comprises rectangular membrane plates.
(A) 3D-SIM of PfGAP50-GFP transfectants (stages IV(i,ii) and V(iii)) showing vertical striations (white arrows). Rotations of the 3D-SIM data are presented in Movies S3. The cells are co-stained with DAPI. Panel Aii is a zoomed view of the cell in Ai, highlighting the periodic striations (arrows). Scale bars = 3 μm. (B) Two 30 nm virtual sections from a cryo electron tomogram of a region near the surface of a gametocyte. (Section (ii) is 100 nm above section (i)). The IMC, parasite membrane (PM) and microtubules (MTs) are marked. The IMC is segmented by vertical striations of amorphous material (Bii), marked as "links". (C) Lying directly underneath, and running perpendicular to the rectangular sections of IMC, is a network of microtubules. Shown are virtual sections of an electron cryo tomogram of a longitudinal section of microtubules and the same section rotated 90ºC to obtain a cross sectional view. Microtubule singlets (s, 25 nm diameter) and doublets (d, 42 nm) are represented. The microtubules are separated by gap (g) of about 10 nm. Scale bars = 100 nm.
Figure 6. Coordinated development of the IMC and the microtubule network drives shape change.

(A) Live cell confocal imaging of stage II - V gametocytes labeled with Tubulin Tracker™ (TT, green) and BODIPY-TR-ceramide (red). DIC images are shown at right. (B) Single sections from a 3D-SIM reconstruction of stage III PfGAP50-GFP gametocytes prepared for immunofluorescence microscopy (anti-GFP, green; anti-β-tubulin, red). The white arrows mark areas of peripheral PfGAP50-GFP staining with associated β-tubulin. The red arrow in panel ii highlights the looping extensions of the IMC with concentrated β-tubulin staining. A montage of projections of (i) is shown in Figure S3. Projections and 3D rotations of (i) and (ii) are presented in Movies S5a and b. (C) A stage III gametocyte labeled with anti-PfGAP45 (green) and anti-β-tubulin (red). This image highlights the restricted location of PfGAP45 within the foot of the gametocyte. Scale bars = 3 μm. See also Movie S6 and S7.

Figure 7. Gametocyte shape changes are accompanied by changes in deformability.

(A) Elongation index values for uninfected RBCs, asexual trophozoites (30-36 hours post invasion) and stage III - V gametocytes were measured using a RheoScan slit ektocytometer at shear stresses from 0-20 Pa. The data represent the means of triplicate measurements ± S.E. and are typical of experiments performed on two separate occasions. The red box highlights the measurements at 3 Pa. (B) Illustrations of stage III - V gametocytes (female). The dimensions were measured from DIC images (mean ± S.E.). (C) Length to width ratio ± S.E. for different developmental stages.