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Review

# The Riveting Cellular Structures of Apicomplexan Parasites

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**Parasitic protozoa of the phylum Apicomplexa cause a range of human and animal diseases. Their complex life cycles – often heteroxenous with sexual and asexual phases in different hosts – rely on elaborate cytoskeletal structures to enable morphogenesis and motility, organize cell division, and withstand diverse environmental forces. This review primarily focuses on studies using *Toxoplasma gondii* and *Plasmodium* spp. as the best studied apicomplexans; however, many cytoskeletal adaptations are broadly conserved and predate the emergence of the parasitic phylum. After decades cataloguing the constituents of such structures, a dynamic picture is emerging of the assembly and maintenance of apicomplexan cytoskeletons, illuminating how they template and orient critical processes during infection. These observations impact our view of eukaryotic diversity and offer future challenges for cell biology.**

## Highlights

The structural features of the Apicomplexa define the phylum and are essential for their complex life cycles. From the apical and basal complexes to the alveoli and microtubule cytoskeleton, these features are required by the parasites.

The cytoskeleton of the apicomplexan parasites drives cell division and morphogenesis as the parasites transform throughout their complex life cycles.

The role of the microtubule cytoskeleton in organizing and potentiating parasite motility is now appreciated.

Significant advances now allow the identification of many of the proteinaceous rivets first postulated by electron microscopists in the 20th century. This has allowed for the careful dissection of the contribution of structural elements to the parasite's life cycles, revealing specific functions and requirements for these components.

## The Cytoskeletal Adaptations of Apicomplexan Parasites

Apicomplexans comprise a diverse phylum of parasitic protists that replicate exclusively within animal hosts. These organisms include the etiological agents of several prevalent human diseases including malaria, caused by *Plasmodium* spp., and eponymous infections caused by *Cryptosporidium* spp. and *T. gondii*. Ultrastructural studies over the past 60 years have defined common morphological features of the phylum, which owes its name to the highly polarized organelles and cytoskeletal structures which dominate the apical end of their motile stages, called zoites (Figure 1A). Characteristic secretory organelles (micronemes and rhoptries) and cytoskeletal structures are organized within the **apical complex** (see Glossary), defined by the presence of the apical polar ring, and occasionally crowned by a cylinder of microtubules named the **conoid**. The apical complex both defines a specialized site of exocytosis and orchestrates the biogenesis of the cell. Either a single (e.g., in *Plasmodium* merozoites) or a tightly sutured series (e.g., *Toxoplasma* tachyzoites) of compressed vesicles (**alveoli**) emerges from the apical complex towards the posterior of the parasite. These subtend the plasma membrane and are supported in turn by a highly stable network of intermediate filaments (the **subpellicular network**, **SPN**) and an array of cortical microtubules. In members of the Apicomplexa, the alveoli and its supportive cytoskeleton are collectively referred to as the **inner membrane complex** (**IMC**) (Figure 1A). Analogous structures, including the alveoli, are present among related free-living alveolates, and subpellicular microtubules can also be found in the single-celled parasitic kinetoplasids [1]. As the IMC and parasite cytoskeleton have assumed critical functions in the infectious cycle of apicomplexans, it warrants study in this important group of pathogens.

Although apicomplexan structural features are broadly conserved, cellular morphology displays significant divergence throughout the parasites' complex life cycles, as summarized in Figure 1B. Apicomplexan parasites undergo a sexual cycle in their definitive host, and many also undergo extensive asexual replication in their intermediate host or hosts. These multiple metamorphoses make the IMC a dynamic structure which is broken down and rebuilt both upon transition between stages (e.g., during *Plasmodium* liver-stage development or gametocytogenesis) and during cell division

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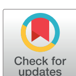


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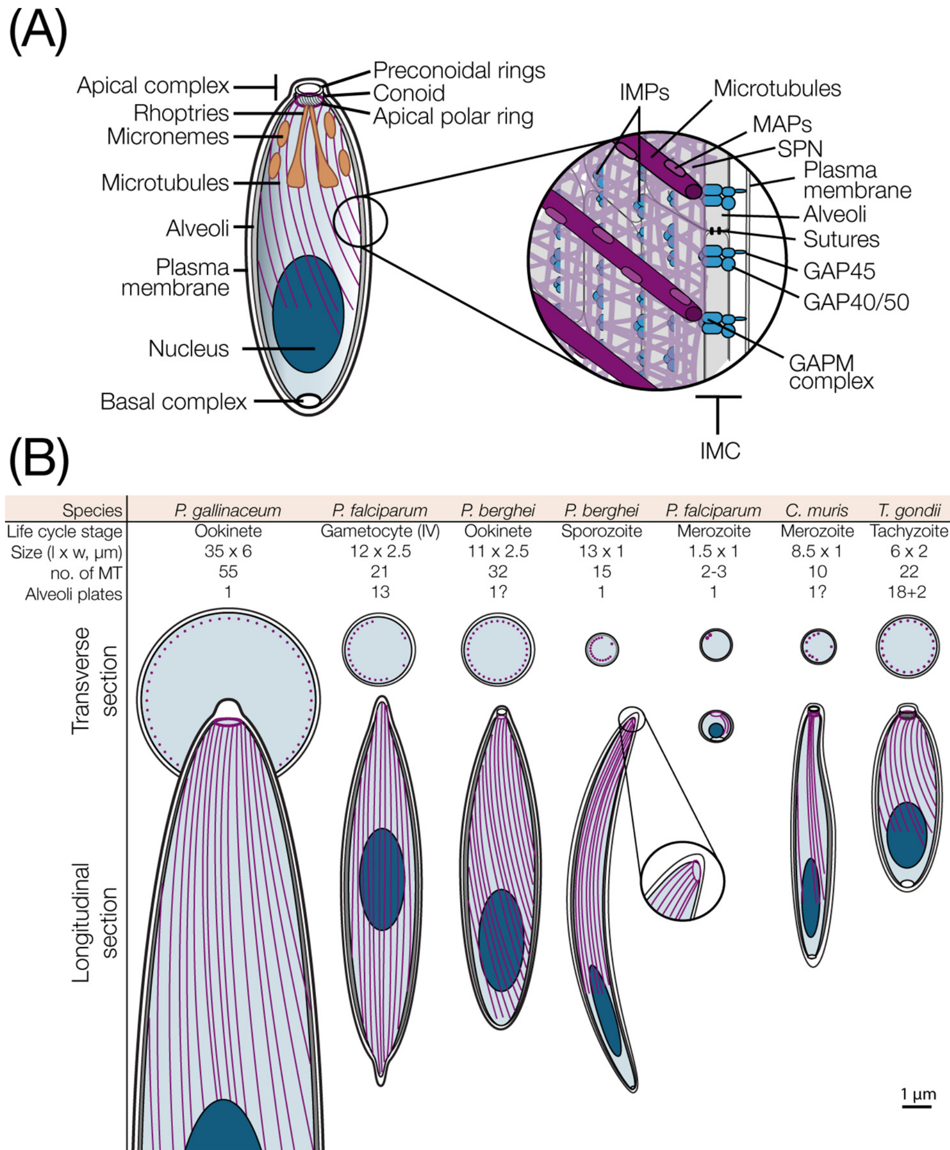


| Species | *P. gallinaceum* | *P. falciparum* | *P. berghei* | *P. berghei* | *P. falciparum* | *C. muris* | *T. gondii* |
|---|---|---|---|---|---|---|---|
| Life cycle stage | Ookinete | Gametocyte (IV) | Ookinete | Sporozoite | Merozoite | Merozoite | Tachyzoite |
| Size (l x w, µm) | 35 x 6 | 12 x 2.5 | 11 x 2.5 | 13 x 1 | 1.5 x 1 | 8.5 x 1 | 6 x 2 |
| no. of MT | 55 | 21 | 32 | 15 | 2-3 | 10 | 22 |
| Alveoli plates | 1 | 13 | 1? | 1 | 1 | 1? | 18+2 |

Figure 1. Comparison of Apicomplexan Morphology and Examples of Disruption of Key Structural Features. (A) A model apicomplexan (a coccidian), demonstrating the structural features discussed in this review. IMC, inner membrane complex; MAP, microtubule-associated protein; SPN, subpellicular network; IMP, inner-membrane particle. (B) Apicomplexan zoites demonstrate significant variation in size, shape, and structural elements, including the number and arrangement of supporting microtubules, alveoli plates, and the presence of a conoid. In addition to *Plasmodium falciparum* and *Toxoplasma gondii,* we show zoites from the bird malaria parasite *Plasmodium gallinaceum* and the mouse malaria parasite *Plasmodium berghei* as well as the mouse diarrheal parasite *Cryptosporidium muris*. Detail shows the tilting of the polar rings in *Plasmodium* sporozoites. Abbreviation: MT, microtubules. Scale bar: 1 µm.

(e.g., asexual division in both *Plasmodium* and *T. gondii*) in order to meet the immediate needs of the parasite. One of the most dramatic cytoskeletal assemblies occurs during male gametogenesis, when a poorly characterized basal body and flagella are made within minutes of activation to generate the only flagellated form of the Apicomplexa [2]. However, impressive cytoskeletal reorganizations can be seen at many of the transitions between life cycle stages.

## Glossary

**Actin–myosin motor complex:** a complex of proteins including actin, the atypical myosin MyoA (and associated light chains) adhesins which stretch across the plasma membrane (e.g., AMA1 and TRAP family members), GAP45 which links the plasma membrane to the alveoli, and anchors (e.g., GAP40, GAP50) which span the membranes of the inner membrane complex, fixing the complex in place. Also known as the glideosome.

**Alveoli:** flattened vesicles, possibly derived from the endoplasmic reticulum (ER) and tightly sutured together, which subtend the plasma membrane in members of the Apicomplexa and the infrakingdom of Alveolata.

**Apical complex:** a structural feature which defines the Apicomplexa; it consists of the apical polar ring which acts as an unusual ring-shaped MTOC, contains specialized secretory organelles, and serves as the focal point for parasite secretion. The coccidians have elaborated on this structure to form the conoid.

**Basal complex:** a contractile ring which develops during daughter cell formation and provides the force required for budding and cytokinesis.

**Conoid:** a complex structure localized at the apical tip of a subset of Apicomplexa; it is made up of the apical polar ring, two preconoidal rings, a number of highly curved tubulin protofilaments, and two intraconoidal microtubules.

**Endodyogeny:** cell division where two daughter cells are constructed within the mother then elongate and emerge, destroying the mother cell in the process.

**Gliding motility:** an unusual form of substrate-based motility during which the cell does not change its shape; it is mediated by the translocation of adhesins down the length of the parasite through the action of an actin–myosin motor.

**Inner membrane complex (IMC):** a structure formed of tightly sutured alveoli, supported on the cytosolic side by the subpellicular network and subpellicular microtubules.

**Inner membranous particles (IMPs):** small proteinaceous particles, observed by scanning electron microscopy (SEM), found on all faces of the alveoli in ordered rows, aligned with both filaments of the SPN and the subpellicular microtubules.

In this review we discuss the multifarious roles of the IMC and cytoskeleton during cell division, stage conversion, and in determining cell shape and polarity (Figure 1B). We also draw on recent work addressing the roles of the IMC in organizing parasite motility and in mitigating the environmental forces that the parasites experience. By studying these processes in the highly divergent Apicomplexa, we gain an appreciation for the diversity of strategies used by single-celled eukaryotes to organize and survive in frequently hostile environments.

## Templating and Transformation

### Parasite Proliferation

Replication within the apicomplexan phylum, and even between life cycle stages, demonstrates a large degree of flexibility [3]. The Apicomplexa are unusual in that nuclear division and daughter cell construction are independently organized and regulated [3,4]. Disrupting daughter scaffold formation either by removing protein components of the alveoli or SPN, or by disrupting microtubule polymerization leads to stalled daughter cell budding, multiple rounds of nuclear division, and a block in cytokinesis [5–9]. Interestingly, work in *T. gondii* has demonstrated that the parasite is capable of a degree of replicative flexibility; mutations of several IMC components have been shown to affect the number of daughter cells formed [10–12], perhaps highlighting the range of replicative modes used through evolution of these parasites. In *Plasmodium*, the asexual blood stage divides by **schizogony**, in which 8–32 daughter cells are constructed. Distinct processes occur during sporozoite construction, when hundreds of sporozoites are formed within the mosquito, and during liver-stage development when thousands of merozoites are constructed in mammalian liver cells. In all cases, nuclei multiply by closed mitosis within the cytosol of the growing parasite and appear to associate with the plasma membrane. Progeny parasites bud from the membrane by seemingly pulling the nucleus and all other organelles into the newly formed cells. By contrast, *T. gondii* tachyzoites build only two daughter cells which bud from the mother cell (**endodyogeny**), while the sexual stages undergo endopolygeny, where multiple daughter cells bud from a single cell [3] (Figure 2).

However, in all life cycle stages of *Plasmodium* and *T. gondii,* the construction of a daughter cell scaffold remains essential in the generation of infectious, polarized daughter cells. Daughter cell construction begins with either a single or multiple rounds of duplication of the centrosomes and the construction of an atypical **microtubule-organizing center** (**MTOC**) [13,14]. In *T. gondii,* this is organized by striated fiber assemblin (SFA), which links the forming daughters and is required for continuation of budding [15]. Shortly after MTOC formation, proteins of the alveoli and SPN can be seen at the forming daughters [7,11,16]. The alveoli appear to be derived from the parasite's endoplasmic reticulum (ER) [17] and it is believed that they are initially responsible for linking microtubules to the developing daughter scaffolds [9,18] while the polymerization of microtubules drives daughter cell elongation [19].

As the daughter cells grow, the alveoli extend and, in some species and life cycle stages, further alveoli plates are added [5,8], although the final number of plates varies significantly between species and life cycle stages [5,9] (Figure 1B). The alveoli are tightly sutured together, and a number of proteins localized to these sutures have been recently identified in both *T. gondii* and *Plasmodium* [5,8,20]. Interestingly, some suture proteins localize specifically to the transverse sutures, while others are found on both transverse and longitudinal junctions [8]. The specificity of these localizations may suggest that some of these proteins have a role in ensuring the correct positioning of the alveoli, hinting at the intriguing possibility that there are fundamental differences between the rows of alveoli plates. Deletion of suture proteins results in defects in daughter cell morphology, demonstrating the importance of correct positioning and the connections between the alveoli and the parasite [8,21]. Identified suture proteins show poor conservation between members

**Microtubule-organizing center (MTOC):** a structure that nucleates microtubules; in members of the Apicomplexa multiple MTOCs exist, including unusual, ring-shaped structures which nucleate the subpellicular cytoskeleton.
**Schizogony:** cell division in which multiple daughter cells are synchronously constructed and bud from the mother cell's plasma membrane.
**Subpellicular network (SPN):** an insoluble cytoskeletal network which supports the alveoli; it is made up of intermediate filament-like proteins, called alveolins, and is required for maintaining parasite tensile strength.

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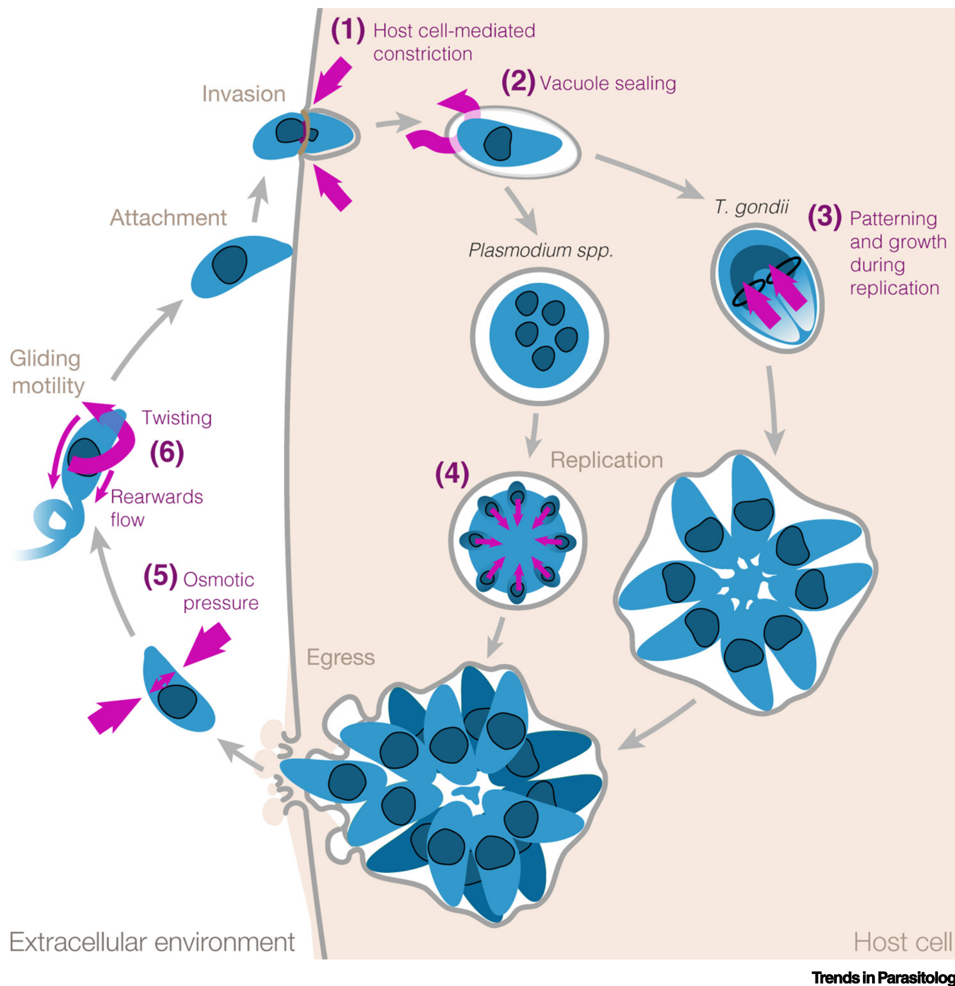


Figure 2. Forces Experienced by Members of the Apicomplexa throughout Their Lytic Life Cycle. A representation of the lytic lifecycle of *Toxoplasma gondii* tachyzoites and *Plasmodium* spp. asexual stages, in pink the forces which the parasite experiences. (1) During invasion, pressure from the host cell actin cortex deforms the parasite, which uses actin to protect its nucleus. (2) At the end of invasion, the parasite can perform a further half twist, presumably to help seal the vacuole. (3) During endodyogeny, the daughter cells grow through first the polymerization of the microtubules and the movement of the basal complex down the daughter cell. (4) During schizogony, the forces required to build daughters are likely similar but not known. After a period of asynchronous nuclear division, the nuclei migrate to the nascent daughter cells which require the basal complex to complete budding. (5) Upon entering the extracellular environment, parasites are exposed to radically altered osmotic conditions. (6) During gliding motility, parasites attach to their substrate through adhesins and can induce a twist which stores kinetic energy, allowing for forward momentum. Adhesions are recycled down the body of the parasite by rearwards flow.

of the Apicomplexa, potentially related to the differing patterning of alveoli between species. Currently, nothing is known about the contents of the alveoli, although they have been hypothesized to act as a calcium store [22]. This will be crucial to understand if these vesicular structures also have a role in signaling, as is the case in the distantly related ciliates [23].

Daughter cell budding is driven by polymerization of the subpellicular microtubules, and blocking polymerization of microtubules leads to stalled daughter cell budding [9]. The arrangement and number of subpellicular microtubules, although fixed within a life cycle stage, varies extensively between species and life cycle stages. For example in *Plasmodium falciparum,* merozoites

contain only two or three microtubules [9], while ookinetes contain over 60 [24] and sporozoites more than 11 [25] (Figure 1B). The arrangement and number of microtubules is presumably dependent on the MTOC; however, how the stereotyped arrangements are patterned across multiple stages of the same species is unknown. Interestingly, the number of microtubules per cell appears to be correlated with surface area across the Apicomplexa [26] and depends on the level of tubulin expression [27]. In *T. gondii* the subpellicular microtubules extend only two-thirds of the way down the parasite, hence, completion of budding requires the **basal complex**. This complex is a contractile ring at the basal end of the IMC, made up of a number of specialized components, including specific alveolins [16,28], myosins [29], and proteins involved in membrane organization such as MORN1 [30]. Several of these components are recruited early in daughter cell formation, forming a ring at the base of extending daughter cells. As the basal complex passes the end of the subpellicular microtubules, it starts to constrict. The constriction of this ring is driven by a specialized actin–myosin motor complex, powered by the myosin MyoJ [29,31]. The basal complex is driven to the base of the newly formed daughter cell; however, the daughter cells remain connected, with a continuous cytoplasm, through the residual body until egress. During egress, the final stage of cytokinesis occurs in a MORN1-dependent manner, releasing the parasites for the next cycle of invasion [30,32]. The basal complex appears to function similarly across the Apicomplexa and there is some conservation between basal complex-localized proteins [30,32]. However, species-specific proteins, such as CINCH, found only in *Plasmodium* [28], and the extension of microtubules down the entire length of budding sporozoites [27], suggest that some proteins and structures have specialized functions, reflecting the differences in cell division between species.

The unusual methods of cell division utilized by the Apicomplexa generally depend on the accurate construction of daughter cell IMC scaffolds and the destruction and partial recycling of the maternal cytoskeleton. Some of the proteins involved in these processes have now been identified; however, much is still unknown about how they are organized and regulated. While commonalities between schizogony and endodyogeny have been identified, less is known about species-specific mechanisms. Further, the identification of components of the alveoli sutures will allow for detailed examination of how the alveoli are assembled during budding, possibly revealing early polarization of the alveoli themselves.

## Demolition and Development

All apicomplexan parasites undergo significant morphological changes throughout their life cycles. These changes have been best studied in *Plasmodium*, where all stages of the life cycle are readily accessible. Starting from the asexual replication cycle, *Plasmodium* differentiates into gametocytes within red blood cells. Upon uptake by a mosquito vector, male gametocytes undergo a dramatic exflagellation process, and male and female gametes fuse and transmute into the motile zygotes, ookinetes. Ookinetes then transition to oocysts, within which hundreds of sporozoites are formed. Sporozoites are released from the oocyst and float to the salivary glands of the mosquito, which they enter. Upon a mosquito bite, these slender parasites then travel through the skin of the host to the liver, where they transform into extraerythrocytic forms (EEFs). Eventually, these release thousands of merozoites into the bloodstream to begin the cycle once again. Many of these dramatic transmutations are driven by the rapid and orchestrated construction or destruction of structural components within the cell, notably components of the IMC.

The maturation of gametocytes has received significant attention, partly due to its perceived attractiveness as an avenue for blocking transmission [33]. Gametocytes differ in structure between *Plasmodium* species, but do not have apical polarity and lack a recognized MTOC.

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Instead, microtubules nucleate in *P. falciparum* from a ring-shaped deposition of alveoli plates which appears on one side of the nascent gametocyte. These plates expand, concordant with, although slightly ahead of, microtubule polymerization, to form the characteristic elongated shape of the gametocytes [34,35]. Integral components are recruited to the alveoli as they expand, including multiple components of the **actin–myosin motor complex** such as GAP45, GAP50, and MyoA, as well as the glideosome associated protein with multiple membrane spans (GAPMs) and PhiL1 [35–37]. Many of these proteins likely serve a structural role, as depletion can block IMC elongation in gametocytes and prevent maturation [35], although the role of MyoA remains opaque as these cells are not motile. In the final stage of gametocyte maturation, the microtubules depolymerize, leading to a small decrease in parasite length and alterations in the deformability of the host red blood cell [35,36,38]. Little is currently known about the mechanisms or regulation of the orchestrated microtubule depolymerization. Microtubules are disassembled both in gametocyte maturation and during cell division. Disruption of the link between the alveoli and the microtubules appears to trigger depolymerization *in T. gondii,* but only in living cells [26], suggesting the presence of factors within the cytoplasm which trigger depolymerization of free microtubules. Regulating microtubule attachment during budding and gametocyte maturation may be important in triggering depolymerization; however, this has not yet been studied in *Plasmodium*.

Gametocytes are not polarized; however, the development of polarity is essential for the next major stage in the life cycle. Once the gametes have fused, they form a zygote which then must differentiate into a motile, polarized ookinete [39]. The zygote-to-ookinete transition is driven by microtubule polymerization and the rebuilding of the IMC, as downregulating IMC components leads to arrest at the zygote stage [40–42]. In the developing zygote, apical polarity is first marked by the alveoli-resident proteins ISP1/3 and a subset of myosins which then recruit other proteins required for motility [43,44]. ISP1/3 rely on palmitoylation for localization by the apically restricted palmitoyl-S-acyl-transferase DHHC2. DHHC2 is present in the cytoplasm prior to transition, whereupon it self-palmitoleates and associates with the nascent alveoli membrane as one of the initial steps in zygote–ookinete transition [42].

The ookinete then differentiates further into the oocyst, which constructs hundreds of sporozoites [25,45]. After multiple rounds of nuclear division, the oocyst plasma membrane forms invaginations into the cell. Nuclei are recruited to the plasma membrane, possibly by a striated fiber, and patches of IMC are formed, leading to initial daughter bud formation [45,46]. Microtubules develop from these buds as they extend and possibly contribute to pulling in the daughter nuclei in concert with the budding of the new cell [27]. Curiously, microtubules are not required for initial budding but provide stability to the budding parasite. Early budding also fails in the absence of the plasma membrane-localized circumsporozoite protein (CSP) [46], although any structural or signaling connection between CSP and the IMC has not yet been identified.

Upon infection of mammalian hepatocytes, the long slender sporozoites must metamorphize into the rounded EEFs. Perhaps surprisingly, host cells are not required to mediate this transformation which can occur *in vitro* [47] and depends on glucose and bicarbonate levels [48], demonstrating that metabolite signaling initiates transformation. In order to permit this dramatic shape change, the alveoli are removed and packaged into vesicles within the cytoplasm [49] through a parasite-driven, autophagic process [50]. Interestingly, patches of the inner membrane complex persist in EEFs, but it is not known if this is required to support the large cells or if complete clearance is simply not required [49,51]. The cell then produces merozoites in a synchronized fashion, reminiscent of sporozoite production, although probably driven by at least partially distinct processes [45].

Comparatively very little is known about stage conversion in *T. gondii* due to the inaccessibility of the feline definitive host. Previously, ultrastructural analysis revealed that *T. gondii* sporozoites are structurally very similar to tachyzoites with a fully formed IMC. This is also seen in mature female gametes; however it appears to be missing from the male gamete [52,53]. Recently it was shown that increasing the availability of linoleic acid, through supplementation and inhibition of downstream metabolism, allows the generation of *T. gondii* sexual stages in mice [54]. This may allow the study of these elusive sexual stages, opening the possibility of examining *T. gondii* metamorphosis for the first time.

The morphology of the parasites, as patterned by the IMC, at each stage is critical for their function and survival. Structural characterization of the sexual stages of *T. gondii*, and how apical polarity is established, especially in the construction of motile zoites, remain open questions.

## Marshalling Motility and Enveloping Entry

### Morphology in Motion

Many life cycle stages of the Apicomplexa are motile in the extracellular environment, which mostly takes the unusual form of **gliding motility**. Motility is essential *in vivo* for both *T. gondii* tachyzoites and *Plasmodium* ookinetes and sporozoites, and was recently demonstrated for the first time in merozoites [55]. Gliding motility is used by the parasite to traverse the environment to find new host cells [56,57], and much of the machinery required for motility also has an important role in invasion.

For productive parasite motility to occur, force must be generated and directed in a highly organized and polarized manner. Motility is dependent on the polarized secretion of adhesins through the apical complex and their recycling or shedding at the basal end [58,59]. Force is generated by an actin–myosin motor complex which translocates the secreted adhesions down the body of the parasite [55,58]. The roles of the motor complex components in generating motility have been extensively studied and reviewed elsewhere [58]. However, once generated, the force must be effectively organized to mediate motility. Here, cell shape appears to have an important role; disrupting normal morphology has been shown to lead to inhibition of directional gliding. In *Plasmodium* ookinetes and sporozoites, as well as in *T. gondii* tachyzoites, mutants which alter the shape of the cells, even to a small degree (e.g., a ~15% decrease in length) have been shown to inhibit or alter specific parameters of motility [21,26,27,60–63]. These effects are amplified when parasite motility is assayed in 3D, possibly due to the increased surface area of the cell in contact with the matrix [26,62].

Parasite motility also depends on the organization of the force generated. Within the past decade it has been established that *T. gondii* tachyzoites and *Plasmodium* ookinetes move in a conserved, left-handed corkscrew through a 3D matrix [62–64]. This handedness mirrors the left-handed twist seen in the subpellicular microtubule cytoskeleton, suggesting a role for microtubules in organizing motility. By exploiting laser-trapped microbeads on the surface of *T. gondii,* a recent study revealed that rearwards translocation occurs only at specific ‘tracks’ on the parasite surface, which had the same periodicity as the underlying microtubule cytoskeleton [65]. Of note, subpellicular microtubules are spaced evenly in *T. gondii* and *Plasmodium* ookinetes*,* while in several other motile zoites, including *Plasmodium* merozoites and sporozoites, they are arranged asymmetrically (Figure 1B; [52,66–68]). Curiously, in *Plasmodium* sporozoites, a single microtubule appears to localize on the dorsal side of the parasite, with most others localizing on the ventral side. While the two sides of the parasites have not yet been distinguished by molecular markers, sporozoites move slower and detach much more frequently from the substrate when gliding on their dorsal side than on their ventral side. This suggests that this curious polarity

must offer some advantage over a uniform distribution of microtubules [64]. The polar rings are arranged perpendicularly to the microtubules in *T. gondii* tachyzoites and *Plasmodium* ookinetes, yet are tilted towards the ventral side in sporozoites [64,69] (Figure 1B). This could direct secretion of microneme-based adhesins specifically to the ventral side, allowing a better maintenance of substrate contact and hence more persistent gliding. How this asymmetry is patterned has not yet been studied but could offer insights into how the microtubules organize the forces involved in migration.

Persistently migrating sporozoites attach at specific points of their ventral surface to the substrate and produce traction forces at their center that overcome stalling forces at the rear, which likely arise from adhesins that keep the parasites attached [70,71]. Hence, gliding of sporozoites proceeds in a stick–slip manner that sees brief high instantaneous speed peaks followed by periods of lower speed [70]. Similar peaks of instantaneous speed have been observed in *T. gondii* tachyzoites [72], supporting a similar mechanism between the species. Recently, our understanding of *T. gondii* motility was also extended through an elegant combination of live-cell imaging and detailed analysis of traction forces. It was shown that tachyzoites can use the actin–myosin motor complex to induce increased curvature of the cortical microtubules [72]. This generates a spring-like force which powers forward motility by the parasite. These data indicate that the microtubule cytoskeleton is important in organizing the forces, which corroborates results showing that mutations which change the number, arrangement, or length of microtubules modulate, although not totally block, motility in *T. gondii* and *Plasmodium* sporozoites and ookinetes [26,27,61]. Further, the ability of parasites to move with disrupted microtubules, albeit with altered parameters [26,27], helps to confirm that microtubules are not essential for force generation per se, but instead have an important role in organizing force generation along the apical–posterior axis, and possibly in storing kinetic energy for motility. The number of microtubules varies across species and life cycle stages (Figure 1B); however, it is not known if the changing number of microtubules has a direct effect on parasite motility.

### Surviving the Squeeze

Apicomplexan parasites must migrate through different tissues which may have evolutionarily constrained their shape. For example, *Plasmodium* sporozoites must traverse the narrow ducts of the mosquito's salivary glands, which limits their width to around 1 μm. Many parasites move through the skin or connective tissue and so face tight meshes of fibers that they must navigate through, requiring both a high level of elasticity and structural rigidity. Invasion of their host cells through a tight junction in the plasma membrane provides a further barrier (Figure 2; [58]). This junction squeezes the parasite, constricting it to around two-thirds of its diameter and significantly deforming the parasite microtubules [73,74]. This constriction is a common feature of apicomplexan invasion and is seen in *T. gondii* tachyzoites as well as in *Plasmodium* merozoites [75] and sporozoites [76], although curiously not in ookinetes [77], which, however, can squeeze dramatically as they migrate through the midgut epithelium [78]. The degree of deformation the parasite undergoes appears to both depend on the stiffness of the host cell plasma membrane and the host cell cytoskeleton [74,79–81]. Recent work in *T. gondii* has indicated that the ability of the parasite nucleus to constrict through the tight junction may be a limiting factor during invasion as the rate of entry frequently slows as the nucleus passes through the constriction [73,74]. It is possible that parasite actin may have a role in protecting the nucleus during this process [74], a phenomenon previously observed in mammalian cell migration [82]. Interestingly, during merozoite invasion of red blood cells (RBCs), the nucleus does not appear to be a limiting factor [55], possibly due to the shape of the merozoite and the relative stiffness of the RBC cortex. The integrity of the pellicle is also likely important in invasion; deletion of the link between the plasma membrane and alveoli in *T. gondii* leads to decreased invasion and the ruffling of membranes at the parasite's

basal end [83], demonstrating the forces applied to the pellicle during invasion. Underlining this, it has been observed that parasites with slower entry kinetics demonstrated rupture of the parasite plasma membrane [80]. These data suggest that invasion may be more traumatic to the parasite than its apparent smooth, rapid progress suggests. There is a twist at the end of internalization as the parasite rotates around its long axis. Twisting likely helps the parasite to seal the parasitophorous vacuole behind it. Failure to do so results in swelling of the nascent vacuole and expulsion and death of the parasite [73,84]. This twisting of internalized parasites appears to be dependent on the invasion machinery.

It has become evident that the parasite's cytoskeleton has an important role in organizing the force generation required for motility and invasion. During the process of invasion, the cytoskeleton further protects the parasite from the forces exerted by the host cell, allowing invasion to proceed smoothly and to help seal the newly created vacuole. Significant advances in biophysical assays, combined with quantitative live-cell imaging and superresolution microscopy, have revealed further details and a previously unappreciated understanding of the forces exerted on the parasite and the role for the microtubule cytoskeleton in parasite motility.

## Stress-resistant Scaffolding

The sudden environmental changes associated with the rapid egress of parasites from the intravacuolar to the extracellular environment, or during transmission from one host to another, presents further formidable challenges to the Apicomplexa. A number of studies have implicated the SPN in maintaining parasite integrity in the face of rapidly changing osmotic conditions [85–87]. The majority of the SPN is made up of intermediate filament-like proteins – named alevolins, based on the presence of characteristic repeats – which are required for correct localization and assembly into a highly insoluble network [16,87,88]. Assembly of the SPN occurs very early during daughter cell construction [16,89] and interestingly, compared to the maternal SPN, the daughter cell SPN can be extracted more easily by detergent, indicating a more fluid composition [90]. This change in solubility may be mediated either by changes in the SPN composition [11] and/or by post-translational modifications of the alveolins [91]. The functional consequences are not yet well understood; however, a more deformable scaffold may be necessary in the constricted environment of the mother during budding. This has proved difficult to investigate due to the redundancy of alveolins present in *T. gondii* where 15 alveolins have been identified [11,16].

For this reason, the roles of the SPN have been most comprehensively examined in *Plasmodium*. *Plasmodium* encodes only eight alveolins with nonredundant roles at specific life cycle stages. Careful dissection of these proteins in *Plasmodium berghei* has shown that the composition of the SPN has a stage-specific role in maintaining the tensile strength of the parasite, as well as a role in morphology [60,85,87,92]. In both *T. gondii* and *Sarcocystis*, IMC proteins undergo a dynamic and hierarchical assembly throughout both endodyogeny and the life cycle [11,16]. In *T. gondii* several alveolins have been shown to have essential roles in tensile strength [11], although no changes in parasite shape were seen, possibly due to a higher degree of redundancy in this organism. From these data, it is likely that the major role of the SPN is to maintain the integrity of the parasite periphery under tension. Interestingly, a reduction of tensile strength, even in the absence of morphological or motility changes, reduces ookinete infectivity within the mosquito, demonstrating the importance of parasite resilience in this environment [87].

In addition to the SPN, the microtubule cytoskeleton of the Apicomplexa has unusual environmental stability and is resistant to depolymerization either from monomer extraction via detergent treatment [93,94] or cold treatment [95,96]. The stability of parasite microtubules appears to depend on multiple factors, including post-translational modifications of tubulin, the presence of

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microtubule-binding proteins (MAPs), including proteins localized within the microtubule lumen, and the anchoring of the tubules to the alveoli [26,93,96,97]. However, while the microtubules are highly stable, recently it was shown that extended contraction by the parasite's motility machinery can cause them to deform and break [72], highlighting the forces involved in parasite motility.

The SPN, together with subpellicular microtubules, are crucial elements in protecting Apicomplexa from the stress of external forces. Unlike many other eukaryotic cells, the Apicomplexa seem to lack a typical actin cortex, and it appears that the SPN has taken on some of these roles. There are still numerous questions about how the SPN is constructed and modified through the life cycle, and why microtubules display such unusual stability. One of the stumbling blocks in understanding the roles of the alveolins was imaging the small, densely packed structures. However recent advances in expansion microscopy has allowed unprecedented localization of individual alveolins and microtubules in the cell [69,72,89]. Determining their precise localization will allow functions for individual proteins to be suggested, informing future research.

## Riveting Stable Structures

The intricate arrangements of the apicomplexan IMC is achieved by molecular rivets: proteins or protein complexes mediating the interactions between disparate structural elements. Within the IMC, freeze-fracture electron microscopy identified the presence of **inner membranous particles** (**IMPs**) over 20 years ago. IMPs are protein complexes on the inner face of the alveoli which were shown to align with both the SPN and the subpellicular microtubules [95,98]. The identity of the rivets between the SPN and the alveoli is not currently known; however, several proteins required to maintain the association between microtubules and alveoli have been identified. This close association is required by the parasites to maintain their morphology. Recently, evidence from *T. gondii* and *Plasmodium* has suggested that a protein complex, comprised of Glideosome associated protein with multiple membrane spans (GAPM) proteins, PhIL1, and other protein components, is responsible for maintaining, although not initiating, this connection [26,35,37,99]. The GAPMs form large, stable protein oligomers in the alveoli which associate with PhiL1 [35,99]. In *T. gondii*, ablation of GAPM proteins leads to alterations in the arrangement of microtubules, preceding microtubule depolymerization and alterations in parasite morphology [26]. The role of GAPMs has not been investigated in *Plasmodium*; however, disruption of PhiL1 blocks gametocyte maturation and ookinete conversion, both of which require association between the microtubules and IMC [5,35]. Supporting the hypothesis that alveoli-localized proteins are required to maintain microtubule stability, recent work in *Plasmodium* showed that palmitoylation of two conserved alveoli proteins, ISP1 and ISP3, is required to maintain microtubule integrity during zygote–ookinete transition [42]. ISP1 and ISP3 are restricted to the apical portion of the alveoli [43,44] and so ISP1/3 may be responsible for initial anchoring, while the GAPM family proteins may be important in maintaining the association throughout the cell body.

The apical complex is a key aspect in maintaining structural integrity of the cytoskeleton [100]. In *Plasmodium* ookinetes, the apically localized protein phosphatase containing kelch-like domains (PPLK) has been identified to mediate the connection of the microtubules to the alveoli, in addition to its role in maintaining the stability of the apical complex [61,101]. Further, the apically restricted alveolins AC9 and AC10 are required to maintain the apical polar ring and the arrangement of cortical microtubules in *T. gondii* [89]. These results are interesting as they show that alveolins can also convey patterning information, which may have implications in how cells are constructed.

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The interaction between the alveoli and the plasma membrane must also be maintained. This appears to require a single protein, GAP45, in *T. gondii* [83,102]. In the absence of GAP45 in extracellular parasites, the plasma membrane pulls away from the alveoli and underlying cytoskeleton, although intracellular parasites demonstrated no morphological changes [83,102]. In *Plasmodium* schizonts, lack of GAP45 did not affect intracellular parasite structure (although it did prevent invasion); however, extracellular merozoites were not observed in this study [103]. This demonstrates the importance of the IMC in maintaining parasite morphology and highlights the change in forces the parasite experiences between the intracellular and extracellular environment.

Several rivets have now been characterized which mediate the interactions between structural elements of the cell. Maintaining these links is crucial to the parasite and it is likely that some connections (e.g., between the alveoli and microtubules) have a number of partially redundant interactions. Characterizing the relative importance of these may be challenging but will have implications in how these divergent cells are constructed.

## Concluding Remarks

The diverse members of the apicomplexan family rely on conserved structural elements to build and modulate their morphology. These structures were identified by early electron microscopy studies, and the molecular linchpins are now being identified by genetic dissection, protein expression, and functional assays. These studies have helped to define roles for the distinct structural features discussed here, in building and maintaining the morphology of apicomplexan parasites. However, many significant questions on how these structures are built and maintained remain (see Outstanding Questions). With the extensive genetic, molecular, and imaging toolbox now available we can seek to answer some of these outstanding questions to reveal the rivets and how they maintain the elaborate structure of these cells.

### Outstanding Questions

Members of the Apicomplexa, especially *Plasmodium*, must rapidly build and destroy structural features as they undergo morphogenesis. How is this process controlled and organized? Especially in cases where expected organizational elements are missing, for example, in gametocyte maturation.

What is the role of parasite morphology in invasion, and is parasite morphology constrained by the encountered environment and/or the host cell type?

Do the alveoli have functions which extend beyond their structural roles within the cell, for example, as signaling platforms or ion storage?

How are microtubules patterned in apicomplexan species and life cycle stages, especially those with asymmetric subpellicular microtubules?


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