Expansion microscopy of apicomplexan parasites

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SUMMARY

Apicomplexan parasites comprise significant pathogens of humans, livestock, and wildlife, but also represent a diverse group of eukaryotes with interesting and unique cell biology. Study of cell biology in apicomplexan parasites is complicated by their small size, and historically this has required the application of cutting-edge microscopy techniques to investigate fundamental processes like mitosis or cell division in these organisms. Recently, a technique called expansion microscopy has been developed, which rather than increasing instrument resolution like most imaging modalities, physically expands a biological sample >4-fold. In only a few years since its development, a derivative of expansion microscopy known as ultrastructure-expansion microscopy (U-ExM) has been widely adopted and proven extremely useful for studying cell biology of Apicomplexa. Here we review the insights into apicomplexan cell biology that have been enabled through the use of U-ExM, with a specific focus on *Plasmodium*, *Toxoplasma*, and *Cryptosporidium*. Further, we summarise emerging expansion microscopy modifications and modalities and forecast how these may influence the field of parasite cell biology in future.

Keywords: U-ExM, *Plasmodium*, *Toxoplasma*, *Cryptosporidium*, cytoskeleton.

Cell biology of apicomplexan parasites.

Protists, or protozoans, are the least well studied of the four Linnaean kingdoms of life but exhibit the overwhelming majority of biological diversity in eukaryotes (Patterson 1999, Burki, Roger et al. 2020). Therefore, these single-celled organisms represent a treasure-trove of cell biology that is waiting to be explored. Apicomplexa are a phylum that mostly consists of intracellular protozoan parasites, that include significant pathogens of humans, livestock animals and wildlife. Typically, apicomplexan parasites have distinct lifecycle stages that invade host cells, undergo asexual replication, undergo sexual replication, and enable transmission to a new host (Morrissette and Sibley 2002, Smith, Walliker et al. 2002, Sibley 2010). The diverse ways that apicomplexan parasites perform these most basic biological functions can help reveal aspects of cell biology that are either unique to the phylum or shared across eukaryotes. Further, aspects of cell biology that are unique to Apicomplexa can potentially be targeted with therapeutics to treat and prevent parasitic disease in humans and animals.

This review will focus on the cell biology of three genera of apicomplexan parasites in particular: *Plasmodium*, a parasite that is transmitted by its mosquito host and causes malaria in humans and animals (Cowman, Healer et al. 2016); *Toxoplasma*, a generalist parasite of warm-blooded animals that can cause disease in both humans and livestock animals (Hill and Dubey 2002); and *Cryptosporidium*, an intestinal parasite that is a significant cause of diarrheal disease in humans (Guérin and Striepen 2020). These three parasites were chosen as the focus of this review as they comprise much of apicomplexan diversity, they all represent significant causes of human disease, and they are the apicomplexan parasites whose cell biology is the most well studied.

Investigation of the cell biology of apicomplexan parasites has been vital for developing our understanding of the most fundamental processes in these parasites, along with identifying novel targets for drugs and defining resistance mechanisms for existing drugs. Despite this, many aspects of the cell biology of apicomplexan parasites are extremely difficult to interrogate because of the small size of these parasites. For example, the nucleus of a blood-stage *Plasmodium* parasite is <1 μm in diameter (Rudlaff, Kraemer et al. 2020), which makes resolving sub-nuclear biology difficult or impossible using conventional light microscopy techniques. Further, the rhoptries, secretory organelles that coordinate host cell invasion, are as narrow as ~30

nm in *Toxoplasma* (Mageswaran, Guérin et al. 2021), making it impossible to individually distinguish each of the 8-12 rhoptries in a tachyzoite without using electron microscopy. Historically, these physical barriers have been a driver for innovation in the biological imaging of parasites, with an impressive array of super-resolution light microscopy (Riglar, Richard et al. 2011, Gras, Jimenez-Ruiz et al. 2019), electron microscopy or tomography (Hanssen, Dekiwadia et al. 2013, Mageswaran, Guérin et al. 2021), and x-ray microscopy (Hanssen, Knoechel et al. 2011) techniques having been utilised to interrogate parasite biology. The downside of these techniques, however, is that they often involve extremely expensive infrastructure, non-commercial reagents, specialised expertise to perform, or some combination of these three.

Expansion microscopy.

In 2015, however, an alternative approach to periodically and incrementally upgrading microscopy infrastructure was developed; expansion microscopy (ExM) (Chen, Tillberg et al. 2015). In principle, expansion microscopy is simple, instead of spending hundreds of thousands of dollars to look closer at your sample, you make it bigger. Since the first ExM protocol was published, dozens of derivatives have been developed, but all are similar in at least four particular ways (Wassie, Zhao et al. 2019). Firstly, a biological specimen is covalently anchored to a polymer solution (Wassie, Zhao et al. 2019). This polymer solution is then turned into a hydrogel, that will expand when deionised water is added or shrink when a solution of high osmolarity is added (such as PBS) (Wassie, Zhao et al. 2019). The sample is processed in a way that physically allows it to expand, such as denaturation or proteinase K digestion (Wassie, Zhao et al. 2019). Finally, the sample will be stained with some kind of fluorescent markers, such as fluorophore conjugated antibodies. Overall, these steps combine to physically expand your biological specimen between 4-fold and >20-fold depending on the protocol used (Truckenbrodt, Maidorn et al. 2018, Gambarotto, Zwettler et al. 2019, Wassie, Zhao et al. 2019, Louvel, Haase et al. 2022, Klimas, Gallagher et al. 2023). While ExM represented a tremendous technical and conceptual breakthrough in the way we think about and approach microscopy experiments, early ExM protocols were not widely adopted by the parasitology community.

Ultrastructure expansion microscopy (U-ExM).

Expansion microscopy became widely adopted within the parasitology community following the first published examples of ExM on apicomplexan parasites, with *T. gondii* being expanded using a protocol named

ultrastructure-expansion microscopy (U-ExM) (Tosetti, Dos Santos Pacheco et al. 2020). U-ExM results in ~4.5-fold expansion of the sample and was specifically developed with isotropic ultrastructural preservation of both cells and organelles in mind (Gambarotto, Zwettler et al. 2019). While not very technically different from other ExM protocols, U-ExM exclusively uses commonly commercially available reagents and is compatible with almost all reagents regularly used in fluorescence microscopy, making it easily deployable for parasite labs around the world. Since the first publication of U-ExM on *Toxoplasma*, a further 28 studies have been published on apicomplexan parasites using this technique in less than three years (see Tables 1-3 for summary of all U-ExM studies in Apicomplexa). These studies have spanned five species (*Toxoplasma gondii, Cryptosporidium parvum, P. falciparum, P. yoelii, P. berghei*)., eight lifecycle stages, and localised over 80 proteins using either protein-specific antibodies or tagged proteins (Tables 1-3). A single study using an ExM technique other than U-ExM has been published on apicomplexan parasites (Pavlou, Touquet et al. 2020), but to date U-ExM has been the favoured technique for groups studying Apicomplexa.

This review will briefly introduce the lifecycles of *Plasmodium*, *Toxoplasma*, and *Cryptosporidium*, and summarise how U-ExM has changed our understanding of the cell biology of these parasites.

Plasmodium

While there are many species of *Plasmodium*, this review will predominantly focus on those that infect humans (especially *P. falciparum*), with some information inferred from studies in rodent-infecting *Plasmodium* spp. (*P. berghei* and *P. yoelii*). *Plasmodium* parasites in the sporozoite stage of the lifecycle are transmitted from their mosquito hosts to their mammalian hosts during a blood meal (Frischknecht and Matuschewski 2017). Sporozoites migrate from the skin to the liver, where they will invade a hepatocyte and replicate asexually to form a hepatic schizont that contains thousands of merozoites (Prudêncio, Rodriguez et al. 2006). Merozoites will egress from the hepatic schizont into the blood stream, where they invade host red blood cells (Cowman, Berry et al. 2012). Following red blood cell invasion, parasites progress through the ring and trophozoite morphological stages before undergoing asexual replication to form a schizont that contains ~20 daughter merozoites (Voß, Klaus et al. 2023). Merozoites will then egress from the red blood cell (Dvorin and Goldberg 2022) to invade a new host red blood cell and repeat this cycle of asexual replication. A portion of these asexual parasites will commit to sexual differentiation (Neveu, Beri et al. 2020) and form

sexual stages known as gametocytes (Josling and Llinás 2015). Male and female gametocytes are transferred to a mosquito through a subsequent blood meal, where they will egress from the red blood cell and undergo gametogenesis (Dash, Sachdeva et al. 2022). Each male gametocyte undergoes three rounds of mitosis to form eight gametes, which will fertilise a female gamete to form a diploid zygote (Guttery, Roques et al. 2015). The zygote will soon undergo meiosis and form a tetraploid ookinete (Guttery, Roques et al. 2015), which will invade the mosquito midgut wall and form an oocyst (Vinetz 2005). Inside this oocyst, the parasite replicates asexually to form tens of thousands of sporozoites (Frischknecht and Matuschewski 2017). Once fully mature, sporozoites will rupture from the oocyst into the haemocoel of the mosquito before migrating to and invading the mosquito salivary gland (Mueller, Kohlhepp et al. 2010). Sporozoites will then reside in the salivary gland until the mosquito takes another blood meal.

Across their lifecycle, malaria parasites perform at least four distinct host cell invasion and egress events, transmission between hosts twice, and undergo asexual replication four times and sexual replication once. These major events are hubs for discovery and investigation of *Plasmodium*-specific, apicomplexan-specific, and broad eukaryote cell biology.

U-ExM in Plasmodium

The first study using U-ExM in *Plasmodium* expanded gametocytes, ookinetes and schizonts of *P. berghei* (Bertiaux, Balestra et al. 2021). While the focus of this study was on the cytoskeleton of the parasites, the authors importantly stained parasites with a fluorophore-conjugated N-hydroxysuccinimide (NHS) ester (Bertiaux, Balestra et al. 2021). NHS ester binds free amine groups and so when parasites stained with NHS ester conjugated to a fluorophore (hereafter simply referred to as NHS ester) are visualised(Nanda and Lorsch 2014), the outcome is essentially a protein density map of the parasite. Use of NHS ester therefore allows the visualisation of a number of protein dense structures within the parasite (similar to electron microscopy), without the need for protein-specific antibodies. Across various stages of the *Plasmodium* lifecycle, NHS ester has been used to visualise the rhoptries, cytoskeleton, basal complex, cytostomes, apical polar ring (APR), and nuclear microtubule organising centre (MTOC) (Bertiaux, Balestra et al. 2021, Liffner and Absalon 2021, Simon, Funaya et al. 2021, Rashpa and Brochet 2022, Liffner, Cepeda Diaz et al. 2023, Rashpa, Klages et al. 2023, Yang, Cai et al. 2023); highlighting it is a powerful tool for generally observing changes within the

parasite. Following publication of the first study using U-ExM in *Plasmodium*, further significant developments were made when the protocol was optimised for the most common *Plasmodium* fixative, paraformaldehyde (Simon, Funaya et al. 2021), followed by the first U-ExM experiments using *P. falciparum* (Simon, Funaya et al. 2021). Subsequently, modifications to the U-ExM protocol were made to streamline the process for harvesting malaria parasites at multiple timepoints/lifecycle stages (Liffner and Absalon 2021), along with the testing of a wide number of marker antibodies and chemical dyes (Liffner, Cepeda Diaz et al. 2023). To date, nearly all lifecycle *Plasmodium* lifecycle stages have been expanded using U-ExM (Figure 2). These experiments have spanned *P. falciparum*, *P. yoelii,* and *P. berghei* and localised dozens of proteins (Table 1). Below, we provide a brief summary of some of the cell biology findings in *Plasmodium* that have been enabled by U-ExM.

The cytoskeleton of *Plasmodium* redefined.

Since the first application of U-ExM to malaria parasites (Bertiaux, Balestra et al. 2021), the cytoskeleton of the parasite has been overwhelmingly the most studied aspect of parasite cell biology. The most significant finding of the first U-ExM study in *Plasmodium* was the conservation of a conoid during the ookinete stage of the parasite lifecycle (Bertiaux, Balestra et al. 2021). The conoid is a tubulin-rich structure at the apical end of the motile/invasive stages of many apicomplexans (Dos Santos Pacheco, Tosetti et al. 2020). Presence or absence of the conoid in apicomplexan parasites was long thought to be a defining feature of two classes of parasites, the Conoidasida (conoid present) and Aconoidasida (conoid lacking) (Dos Santos Pacheco, Tosetti et al. 2020). *Plasmodium* was considered to be part of the Aconoidasida, but using U-ExM, the authors showed not only that *P. berghei* ookinetes possess a conoid-like structure at their apical end, but also that *Plasmodium* homologues of canonical conoid markers localise to this structure (Bertiaux, Balestra et al. 2021). In addition to this foundational observation of conoid conservation, the authors also visualised the cytoskeleton of activated male gametocytes and gametes and merozoites (Bertiaux, Balestra et al. 2021). They showed that in each of these stages, along with ookinetes, that the subpellicular microtubules (SPMTs), cytosolic microtubules thought to define parasite shape, are stabilised by polyglutamylation and that this polyglutamylation can be recognised using anti-PolyE antibodies (Bertiaux, Balestra et al. 2021). The ability

to specifically detect SPMTs has subsequently been utilised in other studies to distinguish intranuclear microtubules from SPMTs (Liffner, Cepeda Diaz et al. 2023).

Once the cytoskeleton of gametocytes and ookinetes had been defined using U-ExM, subsequent studies have built on these observations by characterising cytoskeletal proteins using U-ExM. A recent study using U-ExM in *P. yoelii* showed that Apical Polar Ring protein 2 (APR2) is involved with stabilising ookinete SPMTs and anchoring them to the APR, with APR2 knockout parasites showing microtubule accumulations at their basal end (Qian, Wang et al. 2022). Other studies have shown that F-box protein 1 (FBXO1) (Rashpa, Klages et al. 2023) along with kinesins 13 and 20 (Zeeshan, Rashpa et al. 2022) play a role in cytoskeletal integrity and organisation in both ookinetes and male gametocytes. Collectively, this highlights the importance of the initial description of the *Plasmodium* cytoskeleton by U-ExM as it is now being leveraged as a platform to identify and investigate cytoskeletal proteins and phenotypes.

Description of a bipartite nuclear microtubule organising centre (MTOC).

Apicomplexa undergo closed mitosis (Gerald, Mahajan et al. 2011, Voß, Klaus et al. 2023), with the nuclear envelope remaining intact during mitosis. As a consequence of this, the microtubules that coordinate mitosis are formed from a microtubule organising centre (MTOC) embedded within the nuclear envelope. Similarly to the use of NHS ester to identify the conoid of ookinetes, the coupling of U-ExM and NHS ester was used to define the bipartite nuclear MTOC of *Plasmodium* (Liffner and Absalon 2021, Simon, Funaya et al. 2021). Using U-ExM along with other advanced imaging modalities, it has recently been shown in asexual blood-stage parasites (Liffner and Absalon 2021, Simon, Funaya et al. 2021, Liffner, Cepeda Diaz et al. 2023) and gametocytes (Li, Shami et al. 2022, Rashpa and Brochet 2022) that the nuclear MTOC is comprised of an intranuclear region and a cytoplasmic extension(s). Until the advent of U-ExM, the only marker of the nuclear MTOC for light microscopy were anti-centrin antibodies. Anti-centrin antibodies, and likely all 4 centrin proteins encoded in *Plasmodium*, however, have recently been shown to localise exclusively to the cytoplasmic extension of the bipartite nuclear MTOC (Simon, Funaya et al. 2021, Liffner, Cepeda Diaz et al. 2023, Voß, Klaus et al. 2023).

One finding of the study that first characterised the nuclear MTOC of *Plasmodium* by U-ExM was that microtubules could be observed before centrin (Simon, Funaya et al. 2021). This suggested that centrin was

not required for nuclear microtubule polymerisation, but raised the question of when the nuclear MTOC was formed. A study that tracked a number of *P. falciparum* markers across the asexual blood-stage of the lifecycle, which used NHS ester and centrin as markers for the MTOC, subsequently showed that the intranuclear portion of the MTOC was built before the cytoplasmic extensions (Liffner, Cepeda Diaz et al. 2023). This showed that the intranuclear portion of the MTOC is built before the cytoplasmic extensions, and that only the intranuclear portion is necessary for the nucleation of intranuclear microtubules. Additionally, it was shown that the MTOC is not observable in fully-formed merozoites or newly-invaded ring-stage parasites (Liffner, Cepeda Diaz et al. 2023), suggesting that the MTOC disassembles prior to egress and forms *de novo* following reinvasion.

While the cytoplasmic portion of the nuclear MTOC is not required for making the microtubules that coordinate mitosis, it has subsequently been shown that they nucleate cytoplasmic microtubules (Li, Shami et al. 2022, Rashpa and Brochet 2022, Liffner, Cepeda Diaz et al. 2023). In asexual blood stages, the SPMTs that define merozoites seem to emerge from the cytoplasmic extensions of the MTOC (Liffner, Cepeda Diaz et al. 2023). Further, the SPMTs of both gametocytes and gametes have been shown to emerge from the MTOC cytosolic extensions (Li, Shami et al. 2022, Rashpa and Brochet 2022). Collectively, this highlights the nuclear MTOC as both a bipartite and bifunctional structure, with the intranuclear portion nucleating the microtubules that coordinate mitosis and the cytoplasmic extensions nucleating SPMTs.

<u>The nuclear MTOC as a hub for cellular organisation.</u>

In addition to its potential role in nucleating SPMTs, U-ExM studies have suggested that the cytoplasmic extensions of the nuclear MTOC may act as a global hub for cellular organisation (Liffner, Cepeda Diaz et al. 2023, Rashpa, Klages et al. 2023). Using U-ExM, the cytoplasmic extensions of the MTOC were found in a small region between the nuclear envelope and parasite plasma membrane (PPM) (Liffner, Cepeda Diaz et al. 2023). This led to the suggestion that the nuclei of asexual blood stage parasites are anchored in place to the PPM by the MTOC (Liffner, Cepeda Diaz et al. 2023). Temporally, this seems to occur when parasites start to undergo mitosis, with nuclei appearing anchored to the PPM from the onset of mitosis until part way through segmentation (Liffner, Cepeda Diaz et al. 2023).

When the MTOC first anchors to the PPM, there does not appear to be any other recognisable structures or organelles in the space between the nucleus and PPM. Eventually, however, this small area will go on to define the apical end of the forming merozoites (Liffner, Cepeda Diaz et al. 2023, Rashpa, Klages et al. 2023). Not long after nucleus anchoring, each MTOC is accompanied by a Golgi. Subsequently, the apical polar ring and rhoptries undergo their biogenesis in this small space between the PPM and nuclear envelope (Liffner, Cepeda Diaz et al. 2023). Marking the start of segmentation, the basal complex and inner-membrane complex (IMC) also undergo their biogenesis in this confined space (Liffner, Cepeda Diaz et al. 2023), turning an area of the cell that previously lacked any recognisable structures into the highly organised forming end of a merozoite. Once the parasite has begun segmentation, a branch of both the apicoplast and mitochondrion will closely associate with the cytoplasmic extension of the MTOC and subsequently these organelles will undergo fission (Liffner, Cepeda Diaz et al. 2023). It is tempting to speculate that these interactions anchor the apicoplast and mitochondrion within the forming merozoite, subsequently triggering their fission. Collectively, these events establish the nuclear MTOC as hub, around which merozoites are built. Additionally, as the schizont lacks obvious polarity prior to the anchoring of the nucleus, it is possible that the attachment of the MTOC to the PPM establishes regions of polarity within the schizont that will go on to define the apical end of its merozoites.

Mitosis and meiosis at subnuclear resolution.

Plasmodium parasites undergo closed mitosis (Gerald, Mahajan et al. 2011), where the nuclear envelope does not break down during mitosis, followed by nuclear division, and so with uncondensed chromosomes, so mitosis progress cannot be assessed using DNA dyes. During the replicative stages of the *Plasmodium* lifecycle, the parasite undergoes mitosis or nuclear division in at least 3 distinct ways. Asexual blood-stage parasites undergo schizogony, where nuclei undergo rounds of mitosis followed by nuclear division in a shared cytoplasm (Voß, Klaus et al. 2023). During male gametogenesis, the parasite undergoes three rounds of mitosis without nuclear division, creating an 8n nucleus that undergoes combined nuclear and cell division to form 8 gametes (Matthews, Duffy et al. 2018). It has recently been shown that during sporogony in oocysts, large nuclei likely contain multiple genome copies (Araki, Kawai et al. 2020), but the dynamics of mitosis and nuclear division in this stage of the lifecycle are yet to be elucidated.

One of the major challenges of investigating mitosis and meiosis in *Plasmodium* is a lack of available tools to study these processes. As *Plasmodium* undergoes closed mitosis, and some stages of the lifecycle contain multiple genome copies in a single nucleus, a marker of the nuclear envelope is of clear importance. Prior to U-ExM, however, no markers or stains discernibly and uniformly marked the nuclear envelope. To address this, a lipid stain BODIPY ceramide was applied to parasites following U-ExM (Liffner and Absalon 2021). Using BODIPY ceramide, the authors were able to observe many membranous structures within the parasite but most notably the nuclear envelope (Liffner and Absalon 2021). Leveraging this development, they studied the function of minichromosome maintenance binding protein (MCMBP), where they showed that knockdown lead to aneuploidy and the formation of anaphase chromatin bridges (Liffner and Absalon 2021).

As DNA staining does not indicate mitotic/meiotic stage due to the uncondensed chromosomes of *Plasmodium*, this must be inferred from protein markers. Using U-ExM, a recent study validated a number of kinetochore markers that can be used to infer the position of chromosomes during mitosis and meiosis (Brusini, Dos Santos Pacheco et al. 2022). These markers allowed the authors to visualise chromosomes lining up on the metaphase plate, along with their separation during meiosis and mitosis of both gametocytes and asexual blood stages (Brusini, Dos Santos Pacheco et al. 2022). The ability to infer the position of individual chromosomes and kinetochores will have a significant impact on our understanding of the dynamics of mitosis and meiosis in malaria parasites in future. Further, these observations provide a platform for the characterisation of proteins involved in mitosis and meiosis, with a recent study using U-ExM to show that End-binding protein 1 (EB1) mediates microtubule-kinetochore attachment during mitosis of gametocytes (Yang, Cai et al. 2023).

Of the organisms discussed in this review, U-ExM has been applied to by far the greatest diversity of lifecycle stages in *Plasmodium*. At present, nearly the entire *Plasmodium* lifecycle has been visualised using U-ExM (Figure 2). This exemplifies the adaptability of U-ExM to different sample types, and the transferability of observations between lifecycle stages. A comprehensive list of all proteins localised in *Plasmodium* to date can be found in Table 1.

Toxoplasma

Toxoplasma gondii can infect almost any nucleated cell of any warm-blooded animal but it undergoes sexual replication in the intestine of felines, its definitive host (Tenter, Heckeroth et al. 2000). Animals can be infected by either the cyst or oocyst stage of the lifecycle, cysts contain invasive parasite stages known as bradyzoites, while oocysts contain invasive parasite stages known as sporozoites (Hill and Dubey 2002). When a non-felid animal is infected by an oocyst, the oocyst releases sporozoites, which differentiate into tachyzoites and invade host cells (Carruthers and Boothroyd 2007). Following host cell invasion, tachyzoites can either replicate asexually to form daughter tachyzoites that will egress from the host cell and invade a new host cell, or differentiate into bradyzoites (Lyons, McLeod et al. 2002). Bradyzoites form tissue cysts, and when these cysts are ingested by another non-felid animal the cysts release the bradyzoites to invade host cells, differentiate into tachyzoites and repeat this cycle (Hill and Dubey 2002). When a felid is infected by an oocyst, the sporozoites will differentiate first into tachyzoites and subsequently bradyzoites. Differentiated bradyzoites from the oocyst, or ones that come directly from tissue cysts, can then differentiate into either tachyzoites, or sexual stage gametocytes in the intestinal epithelium of the felid (Lourido 2019). Male and female gametocytes can fuse to form a diploid zygote, which will subsequently undergo meiosis to form a haploid oocyst (Martorelli Di Genova and Knoll 2020). This oocyst will then be shed from the felid to reinitiate the infectious cycle in another animal.

U-ExM in Toxoplasma

Of the three genera of parasites discussed in this review, expansion microscopy has been the most widely applied in *Toxoplasma*, with seven different research groups having published using the technique. Overall, U-ExM experiments on *Toxoplasma* to date have heavily focussed on the parasite cytoskeleton, but more recent studies have started exploring other aspects of parasite cell biology. Importantly for users of U-ExM, and other derivative expansion techniques, the tachyzoite conoid is used as a ‘molecular ruler’ to assess expansion factor because of its highly defined dimensions (Louvel, Haase et al. 2022).

Defining the components and function of the *Toxoplasma* conoid.

The past few years have seen tremendous efforts to define the apical end of *Toxoplasma* tachyzoites in incredible detail, using a variety of imaging modalities (Mageswaran, Guérin et al. 2021, Segev-Zarko, Dahlberg et al. 2022, Sun, Segev-Zarko et al. 2022, Li, Du et al. 2023). At the apical end of a tachyzoite is the conoid, a barrel-shaped structure comprised of angled tubulin fibres and containing a further two intraconoidal microtubules (Dos Santos Pacheco, Tosetti et al. 2020). On its apical end, the conoid is flanked by the preconoidal rings (PCRs), while on its basal end the conoid is flanked by the apical polar rings (APRs) (Dos Santos Pacheco, Tosetti et al. 2020). The APRs form the apex of the parasite's inner-membrane complex, and are where the subpellicular microtubules (SPMTs) that define its shape are nucleated from. When tachyzoites are intracellular the conoid is retracted, but when parasites are extracellular the conoid is extruded, allowing parasite motility and invasion.

From the very first study using U-ExM on *Toxoplasma*, it was clear that it was going to serve as a powerful tool for defining the proteins that comprise the apical end of the parasite, and importantly characterising their mutants. In this study, the authors utilised U-ExM to refine the localisation of a number of apical cap (AC) proteins (Tosetti, Dos Santos Pacheco et al. 2020). In doing so, they immediately recognised the tremendous ability of U-ExM to visualise the parasite cytoskeleton and also to unequivocally define whether the conoid was retracted or extruded. Uniquely, AC9 and AC10 were localised specifically to the space between the SPMTs and their knockdown led to significant cytoskeletal defects (Tosetti, Dos Santos Pacheco et al. 2020). Since this study, 23 proteins have been localised to either the PCRs, conoid, or APRs of *Toxoplasma*, highlighting the utility of this technique for precise protein localisation.

Importantly, the ability to visualise all the different regions at the apical end of tachyzoites gave the ability to characterise mutants that deform these structures. A recent study localised a number of proteins to the different subregions of the tachyzoite apical end, including 5 new PCR proteins, and functionally characterised 10 of them using knockdown parasites (Dos Santos Pacheco, Brusini et al. 2022). Specifically, the authors assessed conoid extrusion using U-ExM and found 7 proteins whose knockdown inhibited parasite motility and conoid extrusion (Dos Santos Pacheco, Brusini et al. 2022). Curiously, the authors also characterised homologues of two of these proteins in *P. berghei* ookinetes, and showed that both localise at

the conoid, with knockdown inhibiting ookinete motility and decreasing the distance between the conoid and APR; a likely equivalent of inhibiting conoid extrusion (Dos Santos Pacheco, Brusini et al. 2022). Collectively, using U-ExM the authors defined a new hypothetical model for the process and function of conoid extrusion. When the conoid is extruded, actin flux occurs from the apical end the conoid and accumulates between the parasite plasma membrane (PPM) and inner-membrane complex (IMC) before moving to the basal end of the parasite to generate its actin-based motility. When the conoid is retracted, the PCRs prevent the flux of actin through the space between the PPM and IMC, therefore inhibiting actin-based motility and making conoid extrusion essential for motility (Dos Santos Pacheco, Brusini et al. 2022).

The flexibility of visualising a range of conoid-localising proteins using antibodies or tags, along with the ability to assess changes to the conoid by U-ExM, demonstrate the usefulness of U-ExM for investigating the role of proteins that define parasite ultrastructure.

A closer view of the centrosome and kinetochore of *Toxoplasma*.

Investigating mitosis in *Toxoplasma* is challenging for many of the same reasons as described previously for *Plasmodium.* During the tachyzoite stage of the lifecycle, *Toxoplasma* divides through a process called endodyogeny. In brief, the genome of a mother parasite will duplicate and undergo closed mitosis, with nuclear fission partitioning sister nuclei into two daughter cells that form within the mother (Gubbels, Keroack et al. 2020). During mitosis, and the early stages of daughter cell formation, the two forming daughter buds can be difficult to distinguish from both each other, and the mother parasite; complicating study of *Toxoplasma* mitosis by light microscopy.

Two separate studies have shown that using U-ExM and a combination of antibodies against tubulin and markers of either the centrosome or kinetochore, the stages of mitosis in tachyzoites can be precisely defined (Brusini, Dos Santos Pacheco et al. 2022, Tomasina, Gonzalez et al. 2022). By observing these stages of mitosis, the authors of each study were then able to characterise localisation of proteins during mitosis and characterise their role in mitosis.

The first of these studies focussed on describing new markers of the kinetochore and their relative positions during mitosis (Brusini, Dos Santos Pacheco et al. 2022). Incredibly, the authors were not only able

to distinguish between markers of the kinetochore and centromeric proteins, but also able to define the position of proteins in sub-kinetochore compartments (Brusini, Dos Santos Pacheco et al. 2022). As the alignment of chromosomes along the metaphase plate, and their subsequent separation, could now be visualised, the authors subsequently characterised knockdown mutants of apicomplexan kinetochore protein 1 (AKiT1) and centromere protein C (CENP-C). Following either AKiT1 or CENP-C knockdown, it could be seen that chromosomes fail to properly align along the metaphase plate and then fail to separate appropriately during anaphase (Brusini, Dos Santos Pacheco et al. 2022). The ability to characterise the function of proteins involved in mitosis at this specificity represents a tremendous advance in parasite cell biology and will undoubtedly lead to a significant increase in our understanding of parasite mitosis in the future.

The tachyzoite centrosome is comprised of an outer core that contains two centrioles and a centriole-free inner core, which opposes a nuclear envelope elaboration called the centrocone. The second of these two studies assessed the role of the centrosome inner core on mitosis (Tomasina, Gonzalez et al. 2022). Using U-ExM, the authors showed that the only known marker of the inner core Cep250L1 gets duplicated and separated during mitosis. Subsequently, they generated Cep250L1 knockdown parasites and showed that upon Cep250L1 depletion, parasites have a reduced ability to form their mitotic spindle (Tomasina, Gonzalez et al. 2022). This suggests that the inner core of the tachyzoite centrosome, which previously had no known function, is involved in regulating mitotic spindle assembly. Collectively, these two studies highlight the utility of U-ExM to study parasite mitosis, and functionally characterise proteins involved in the process.

Visualising interactions between organelles.

The organelles within a parasite are not static or standalone structures, instead they are constantly morphing and interacting with each other. Investigating inter-organelle interactions, principally mediated by membrane contact sites, is a relatively but rapidly growing new area of parasite cell biology. A recent study investigated the interaction between the inner-membrane complex and mitochondrion of tachyzoites using U-ExM (Oliveira Souza, Jacobs et al. 2022). In intracellular parasites, the mitochondrion typically adopts a 'lasso-like' shape around the nucleus and knockout of an outer mitochondrial membrane protein known as lasso maintenance factor 1 (LMF1) has been shown disrupt this shape (Jacobs, Charvat et al. 2020). Surprisingly, when the authors looked for LMF1 interactors, their top hits were proteins of the inner-

membrane complex including IMC10 (Oliveira Souza, Jacobs et al. 2022). Using U-ExM, the authors showed that LMF1 localises in close proximity to the IMC, appearing to form a bridge between the mitochondrion and IMC (Oliveira Souza, Jacobs et al. 2022). Subsequently, the authors showed that IMC10 knockdown also results in defects to mitochondrial morphology (Oliveira Souza, Jacobs et al. 2022). Assessing the interaction between LMF1 and the IMC during daughter cell formation using U-ExM, the authors suggested that LMF1 and the IMC interact cytokinesis to uniformly distribute mitochondria around the nuclei of daughter cells and form the characteristic lasso shape (Oliveira Souza, Jacobs et al. 2022). This study demonstrates the power of U-ExM to interrogate not only single organelles, but the dynamic interactions between different organelles.

Toxoplasma holds a unique position as the first expanded apicomplexan parasite, with the researchers who pioneered this technique in *Toxoplasma* tremendously demonstrating how U-ExM can unlock new avenues to explore in parasite cell biology. A comprehensive list of all proteins localised in *Toxoplasma* to date can be found in Table 2.

Cryptosporidium

Unlike both *Toxoplasma* and *Plasmodium*, *Cryptosporidium* can undergo its whole life cycle in the same host. *Cryptosporidium* spp. infect a wide range of animals, but this review will focus on those that infect mammals, which include the two major human pathogens *C. parvum* and *C. hominis*. *Cryptosporidium* is transmitted when a host ingests oocysts (Guérin and Striepen 2020). Oocysts contain sporozoites, which will be released from the oocyst and invade the intestinal epithelium (Guérin and Striepen 2020). Once the sporozoite has invaded an enterocyte it will form a trophozoite, which will replicate asexually to form a meront containing 8 merozoites (Guérin and Striepen 2020). Merozoites will egress from the infected cell, reinvade a new cell and repeat this cycle two times (Guérin and Striepen 2020, English, Guérin et al. 2022). During the third round of asexual replication, merozoites commit to sexual differentiation and following meront egress, the merozoites will invade a new host cell to form either a male or female gamont (Tandel, English et al. 2019, English, Guérin et al. 2022). Male gametes undergo four rounds of mitosis to produce 16 gametes, while female gamonts differentiate into a single gamete (Guérin and Striepen 2020). Subsequently these gametes fuse to form a diploid zygote that will undergo meiosis and form an oocyst (Guérin and Striepen 2020).

Oocysts can either be shed to infect a new host, or undergo auto-infection within the same host, repeating this cycle.

Relative to our understanding of cell biology in *Toxoplasma* and *Plasmodium*, the investigation of *Cryptosporidium* cell biology is still in its infancy. In large part, this is due to the fact that until relatively recently culture, cryopreservation, and genetic manipulation of *Cryptosporidium* were either difficult or not possible (Guérin and Striepen 2020). In recent years, however, genetically tractable, and culturable systems for *Cryptosporidium* have been developed (Vinayak, Pawlowic et al. 2015, Heo, Dutta et al. 2018, Jaskiewicz, Sandlin et al. 2018, Sateriale, Šlapeta et al. 2019, Wilke, Funkhouser-Jones et al. 2019). Consequently, we have seen a long-overdue explosion in our understanding of *Cryptosporidium* biology. In only the last few years, an impressive array of microscopy techniques including live cell microscopy (Guérin, Roy et al. 2021, English, Guérin et al. 2022), structured illumination microscopy (SIM) (Choudhary, Nava et al. 2020, Guérin, Roy et al. 2021), stimulated emission depletion (STED) microscopy (Guérin, Roy et al. 2021), cryo-electron tomography (Mageswaran, Guérin et al. 2021), and most recently U-ExM (Guérin, Strelau et al. 2023), have been employed to better understand *Cryptosporidium* cell biology.

U-ExM in Cryptosporidium

Visualising the spatial proteome of *Cryptosporidium*.

A significant advance in our understanding of *Cryptosporidium* biology was achieved with the recent publication of the spatial proteome of *C. parvum* (Guérin, Strelau et al. 2023). Using a technique known as hyperplexed localisation of organelle proteins by isotope tagging (hyperLOPIT) (Christoforou, Mulvey et al. 2016), the authors localised 1,107 proteins to different organelles or structures within *Cryptosporidium* sporozoites. The output of hyperLOPIT clusters proteins based on fraction extraction profiles, as proteins in the same organelles will have similar extraction profiles. Proteins in the dataset with known localisations can then be used to identify which organelles or structures the observed clusters represent. To validate this data, the authors picked proteins predicted to localise to the micronemes or rhoptry neck and performed colocalisation with known markers for these organelles (Guérin, Strelau et al. 2023). In addition, the authors performed U-ExM on sporozoites coupled with NHS ester staining, which clearly revealed the rhoptry, conoid, crystalloid body and dense granules (Figure 1). The use of U-ExM coupled with NHS ester was proved

invaluable, as previously there were no antibodies that could be used as markers for either crystalloid body or dense granules. This allowed the authors to validate four new dense granules proteins and one new crystalloid body protein (Guérin, Strelau et al. 2023). Use of NHS ester with U-ExM in *Cryptosporidium* is likely to continue to be extremely useful in future, due to the relative dearth of marker-specific antibodies that are commonly or commercially available.

Identification of two secretory organelles in *Cryptosporidium*, dense granules and small granules.

Based on the hyperLOPIT data, the authors observed two clusters of proteins distinct from dense granule clusters predicted to be secreted that contained no marker proteins, and tagged a protein from each of these clusters to investigate their localisation (Guérin, Strelau et al. 2023). Using U-ExM coupled with NHS ester, the authors showed that both proteins were found in several small vesicles near the nucleus that were distinct from the dense granules. This organelle was termed the small granules, and was subsequently shown to be secreted following host cell invasion (Guérin, Strelau et al. 2023). Curiously, some proteins predicted to localise to the small granules proteins are conserved in other Apicomplexa, but whether other Apicomplexa have these secretory organelles is not yet known. Collectively, this highlights the utility of U-ExM not only for performing detailed colocalisation studies, but also highlights its ability to reveal structures previously not visible by conventional light microscopy.

Due to the power of U-ExM for identifying organelles without protein-specific stains, it is likely that of the three genera of parasites discussed in this review, U-ExM has the greatest ability to lead to significant advances in our understanding of *Cryptosporidium* biology. A comprehensive list of all proteins localised in *Cryptosporidium* to date can be found in Table 3.

Observed drawbacks and limitations of U-ExM in Apicomplexa.

Like any imaging modality, U-ExM is accompanied by some limitations and artefacts. It is important, however, for early adopters of U-ExM to recognise these limitations and artefacts and share these findings with the community. To date, the most notable limitation occurs in blood-stage malaria parasites that biomineralize haem into a crystal called haemozoin (Matz 2022). The large haemozoin crystal in the centre of the parasite either does not expand, or does not get anchored to the gel, leaving a large empty space in the

food vacuole where it previously resided (Liffner, Cepeda Diaz et al. 2023). Considering this, any observations about the parasite food vacuole should be made with caution as we know it has been altered a non-physiologically relevant way. Curiously, it has also been observed that non-specific antibody fluorescence accumulates in the region of the cell that used to contain the haemozoin crystal (Liffner, Cepeda Diaz et al. 2023).

To date, U-ExM in apicomplexan parasites has only been used to study cultured cells, but U-ExM is applicable to tissues or whole organs. Considering this, we will likely in future see publications that visualise parasites *in situ*, performing U-ExM on intestinal sections containing *Toxoplasma* or *Cryptosporidium*, or whole expanded salivary glands or midguts from *Plasmodium* infected mosquitoes. One significant challenge this is likely to impose is that it will significantly increase the depth into the gel that you need to image to capture your sample. In our experience, the sample is always brightest at the surface of the gel closest to the objective lens and becomes progressively dimmer the deeper into the gel you image; which is to be expected as working distance increases. While imaging of parasites *in situ* will likely be possible irrespective of this limitation, it will prevent imaging of entire organs or tissues using conventional confocal or widefield microscopy. Imaging modalities such as light sheet or lattice light sheet fluorescence microscopy (Stelzer, Strobl et al. 2021), where the sample is more evenly illuminated could likely overcome this hurdle and provide high-resolution expanded images of tissues or organs infected with parasites.

While U-ExM is extremely useful for revealing previously indistinguishable structures, it is likely not compatible with absolute fluorescence quantification. Absolute fluorescence quantification (where two different samples are compared to each other), like in colocalisation studies for example, is complicated because of the variables that need to be controlled for. In U-ExM, there is a small difference between the expansion factors of gels, with reported ranges of between 3.9 and 4.3-fold expansion and currently (Gambarotto, Zwettler et al. 2019, Bertiaux, Balestra et al. 2021, Liffner and Absalon 2021, Liffner, Cepeda Diaz et al. 2023), there is no way to control for this variance. Compounding this problem is the previously mentioned issue about cells deeper into the gel not being as bright as those at the imaging surface. Further, it is currently unclear how much gel-to-gel variation there is in fluorescence intensity, or whether fluorescence intensity is influenced by position within the gel.

While >80 proteins have been localised in Apicomplexa by U-ExM using either protein-specific antibodies or tagged proteins (Tables 1-3), authors have described not being able to localise a small number of proteins using U-ExM. For U-ExM, the sample is completely denatured and so antibodies will be encountering linear epitopes, while in conventional immunofluorescence assays the antibodies would typically encounter conformational epitopes. Considering this, it is unsurprising that some proteins that are readily localised using conventional immunofluorescence assays can sometimes not be localised using U-ExM. To the best of our knowledge, only two apicomplexan proteins have been published as not being localisable by U-ExM; MCMBP in *P. falciparum* (Liffner and Absalon 2021) and CCP1 in *Cryptosporidium* (Guérin, Strelau et al. 2023).

Potential future applications and emerging expansion microscopy techniques.

U-ExM has already had a drastic influence on our understanding of cell biology in apicomplexan parasites, but expansion microscopy is a rapidly evolving technique. Additionally, due to its relatively recent application to apicomplexan parasites, there are some well-studied aspects of parasite cell biology that have not yet been interrogated using U-ExM. In the final few paragraphs of this review, we will summarise emerging expansion microscopy techniques and speculate on areas of apicomplexan cell biology were U-ExM could provide breakthroughs.

Expanding even further.

The original expansion protocol and its derivatives, including U-ExM, result in approximately 4-fold linear expansion of the sample (Chen, Tillberg et al. 2015, Gambarotto, Zwettler et al. 2019, Wassie, Zhao et al. 2019). More recently, however, protocols have been developed that substantially increase the expansion factor from 10 to ~25-fold linear expansion (Truckenbrodt, Maidorn et al. 2018, M'Saad and Bewersdorf 2020, Damstra, Mohar et al. 2022, Louvel, Haase et al. 2022, Klimas, Gallagher et al. 2023, Shaib, Chouaib et al. 2023). This >4-fold expansion has been achieved in a number of ways including using more swellable hydrogels (Damstra, Mohar et al. 2022), and performing iterative expansion where an already expanded hydrogel is expanded again (M'Saad and Bewersdorf 2020, Louvel, Haase et al. 2022). An iterative expansion microscopy technique known as iterative U-ExM (iU-ExM) has already been applied to *T. gondii*, where the individual microtubules that comprise the conoid were observed; something previously only seen using

electron microscopy (Louvel, Haase et al. 2022). These next-generation expansion microscopy techniques will likely be extremely useful for answering questions about highly protein dense structures not readily resolved by U-ExM, such as the conoid. 25-fold linear expansion, however, corresponds to >10,000-fold volumetric expansion, which would decrease protein concentration so much that many proteins/stains may become impossible to visualise. We think that iU-ExM, and other expansion techniques that expand beyond 4-fold will be extremely useful for determining whether there is subcompartmentalisation of proteins within protein-dense structures. Considering this, some logical candidates to investigate using these techniques are the basal complex, apical polar rings, nuclear MTOC, and nuclear pore complexes.

Multiplexing expansion microscopy.

Perhaps the most exciting avenue for future development of expansion microscopy-based techniques is the ability to combine ExM with other kinds of experiments. Fluorescence *in situ* hyrbidisation (FISH) experiments have been a mainstay of nucleic acid biology for many years. Protocols have been developed that enable both DNA (Klimas, Gallagher et al. 2023) and RNA (Chen, Wassie et al. 2016) FISH on expanded samples, opening the possibility of studying individual genomic loci or RNAs on expanded parasites. Omics technologies have also recently been combined with expansion microscopy, with protocols recently published to perform *in situ* spatial transcriptomics (Alon, Goodwin et al. 2021) and proteomics(Li, Sun et al. 2022) on expanded samples. These techniques are not yet at the level of single-cell transcriptomics or proteomics, but we can imaging this kind of technique being applied to decipher a single-cell transcriptome or proteome of something like the *P. vivax* hypnozoite.

Nature vs nurture.

The last few years have seen the publication of groundbreaking studies that allow for the *in vitro* production of infectious *Plasmodium* sporozoites (Eappen, Li et al. 2022) and *Toxoplasma* cat-restricted sexual stages (Antunes, Shahinas et al. 2023). U-ExM could be used to compare the ultrastructure of these *in vitro* generated parasites against their *in vivo* generated counterparts to define the ways in which they are both similar and different. The advantage of U-ExM over electron microscopy in this context, would be the ability to samples hundreds of cells in three-dimensions, at different stages of their development/differentiation, and do so with antibodies against proteins of interest.

Reimagining invasion.

Host cell invasion is one of the most well studied processes in apicomplexan cell biology but to the best of our knowledge, there are no published examples of expanded parasites in the process of invading. It isn't hard to imagine taking U-ExM and revisiting one of the seminal works on host cell invasion using light microscopy or electron microscopy (such as Riglar, Richard et al. 2011 for *Plasmodium* or Del Rosario, Periz et al. 2019 for *Toxoplasma*). To date, U-ExM has not significantly altered our view of host cell invasion by apicomplexan parasites, but we think this is very likely to change in the near future.

Expanding the world.

This review has focussed primarily on studies in *P. falciparum*, *P. berghei*, *P. yoelii*, *T. gondii* and *C. parvum* largely because there are well-established culture systems for these organisms. Perhaps the biggest strength of U-ExM, however, is that when it is coupled with general stains such as NHS ester or BODIPY ceramide, we can learn a great deal about the cell biology of organisms we cannot genetically manipulate and for which we lack protein-specific antibody. In 2023, a groundbreaking and ambitious study known as Traversing European Coastlines (TREC), led by EMBL and the Fondation Tara Océan, began exploring coastal ecosystems using a wide array of scientific techniques (Ghosh 2022). One of the projects within TREC, led by a team across EMBL, EPFL and University of Geneva, is to profile diversity of plankton ultrastructure using cryo-ExM and U-ExM, combined with other imaging modalities such as volume electron microscopy (Ghosh 2022). In principle, this project plans to explore eukaryotic diversity and take the study of cell biology beyond the walls of a laboratory. Applying a similar thought process to apicomplexan parasites, one could imagine using U-ExM to look at the ultrastructure of malaria parasites inside field-caught mosquitoes, haemosporidian parasites in blood samples from wildlife, or *Toxoplasma* cysts from livestock animals. The future of expansion microscopy, and what that means for that will mean for the study of parasite cell biology is truly exciting.

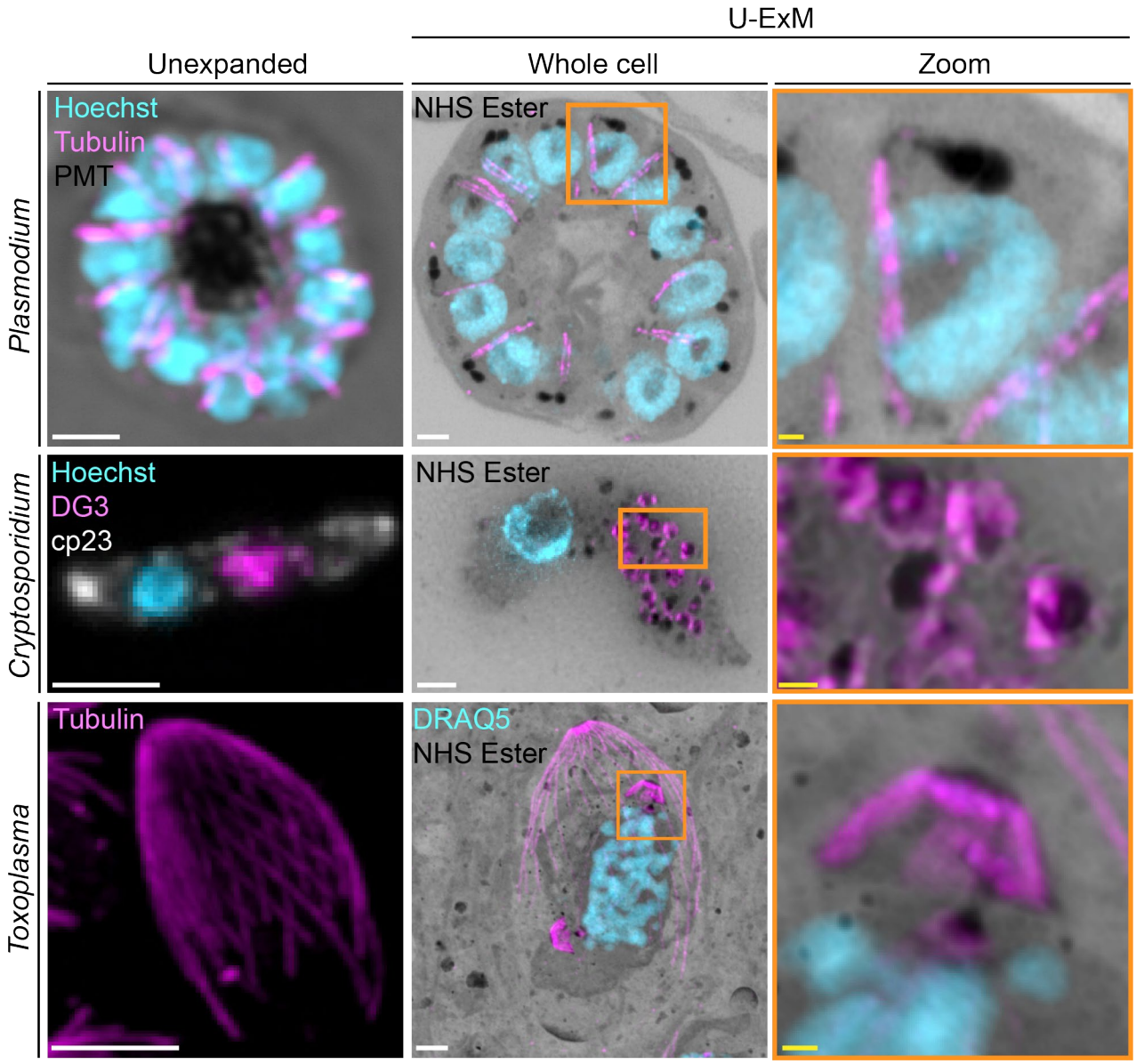

Figure 1: Comparison of unexpanded and U-ExM parasites across Apicomplexa.

Example images of *Plasmodium falciparum* schizonts, *Cryptosporidium parvum* sporozoites and *Toxoplasma gondii* tachyzoites that are either unexpanded, or prepared using U-ExM. For all images DNA stains are represented in cyan. For all U-ExM images, greyscale represents NHS ester (protein density). For *Plasmodium*, magenta represents anti-tubulin (microtubule) staining and zoom region of interest shows a single merozoite from a schizont. For *Cryptosporidium*, magenta represents the dense granule protein DG3, white (unexpanded only) represents the sporozoite antigen Cp23 and zoom region of interest shows individual dense granules. For *Toxoplasma*, magenta represents anti-acetylated tubulin (microtubule) staining and zoom region of interest shows a forming daughter cell. White scale bars = 2 µm, yellow scale bars = 500 nm. *Plasmodium* images are derived from the dataset in Liffner, Cepeda Diaz et al. 2023, *Cryptosporidium* images are unpublished images derived the dataset in Guérin, Strelau et al. 2023, and *Toxoplasma* images are from an unpublished dataset.

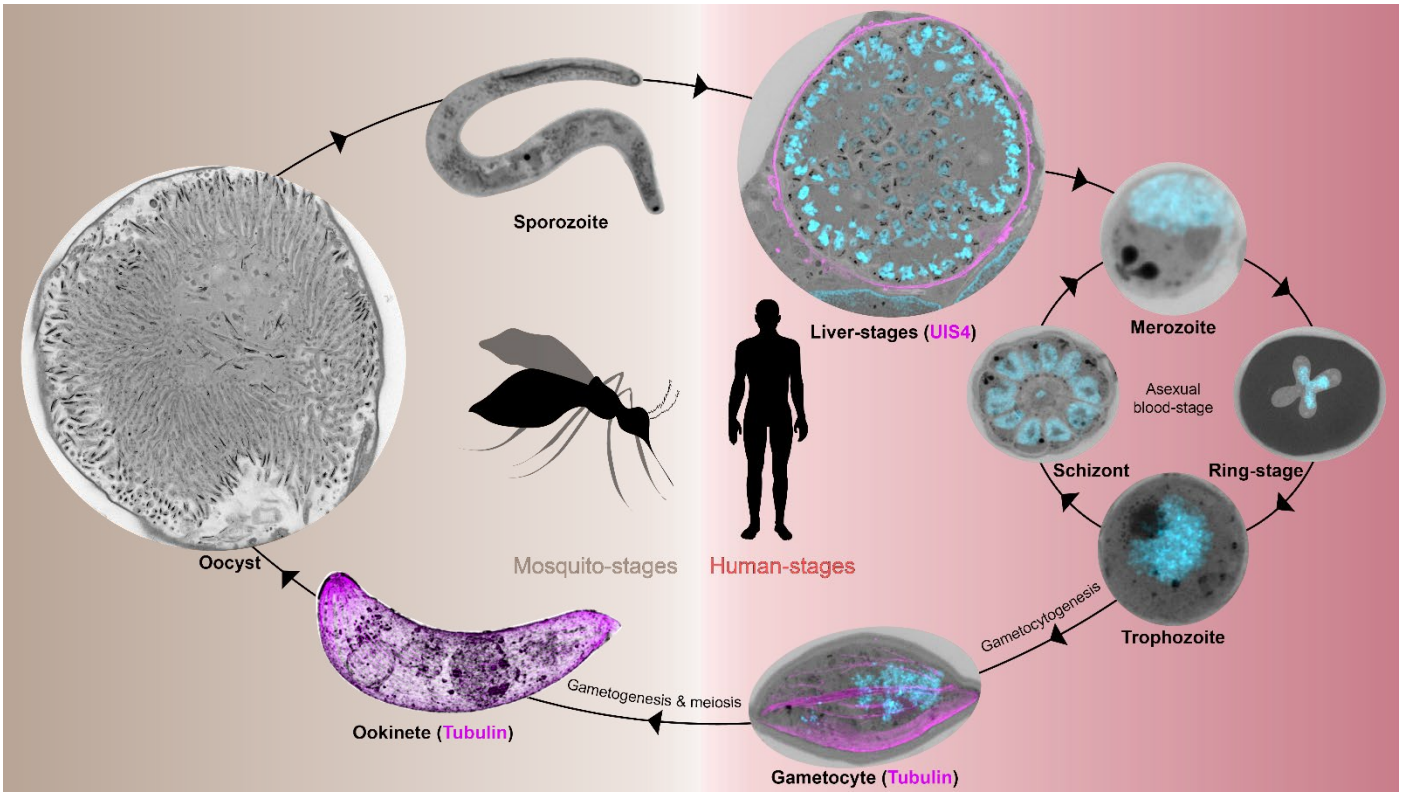

Figure 2: An expanded view of the *Plasmodium* lifecycle.

Generalised *Plasmodium* lifecycle with all represented stages depicted as expanded parasites. For all images greyscale represents protein density (NHS ester) and cyan represents DNA staining (SYTOX deep red). For liver-stages, magenta represents UIS4 (parasitophorous vacuole). For ookinete and gametocyte, magenta represents microtubules. Oocyst and sporozoite images are *P. yoelii* (currently unpublished dataset). Ookinete (replicated with permission from Bertiaux, Balestra et al. 2021) and liver-stages are *P. berghei* (currently unpublished dataset). Asexual blood-stage (from dataset in Liffner, Cepeda Diaz et al. 2023) and gametocyte (currently unpublished dataset) are *P. falciparum*. Images are not depicted to-scale.

Organism	Lifecycle stage	Antibodies and tagged proteins (subcellular location)	References
Plasmodium falciparum	Asexual blood stages	Tubulin, PolyE (microtubules)	(Bertiaux, Balestra et al. 2021, Liffner and Absalon 2021, Simon, Funaya et al. 2021, Liffner, Cepeda Diaz et al. 2023)
		Centrin (MTOC)	(Bertiaux, Balestra et al. 2021, Liffner and Absalon 2021, Simon, Funaya et al. 2021, Liffner, Cepeda Diaz et al. 2023)
		BiP (ER)	(Liffner and Absalon 2021, Liffner, Cepeda Diaz et al. 2023)
		ERD2 (Golgi)	(Liffner, Cepeda Diaz et al. 2023)
		MitoTracker, ATPd (mitochondrion)	(Liffner, Cepeda Diaz et al. 2023)
		ACP^{TS} (apicoplast)	(Liffner, Cepeda Diaz et al. 2023)
		Aldolase (cytoplasm)	(Liffner, Cepeda Diaz et al. 2023)
		CINCH (basal complex)	(Liffner, Cepeda Diaz et al. 2023)
		IMC1g, GAP45 (IMC)	(Liffner, Cepeda Diaz et al. 2023)
		MSP1-19 (PPM)	(Liffner, Cepeda Diaz et al. 2023)
		Kelch13 (cytostomes)	(Liffner, Cepeda Diaz et al. 2023)
		RAP1, RON4, RAMA (rhoptries)	(Liffner, Cepeda Diaz et al. 2023)
		EBA175, AMA1 (micronemes)	(Liffner, Cepeda Diaz et al. 2023)
	Gametocytes/ gametes	Tubulin (microtubules)	(Rashpa and Brochet 2022)
		Actin	(Rashpa and Brochet 2022)
Plasmodium yoelii	Asexual blood stages	CDPK1, MSP1 (PPM)	(Qian, Wang et al. 2022)
	Ookinetes	Tubulin, PolyE (microtubules)	(Qian, Wang et al. 2022)
		SA-488 (APR)	(Qian, Wang et al. 2022)
		APR2 (SPMTs/APR)	(Qian, Wang et al. 2022)
	Gametocytes/ gametes	Tubulin, PolyE, EB1 (microtubules)	(Yang, Cai et al. 2023)
		NDC80 (kinetochore)	(Yang, Cai et al. 2023)
Plasmodium berghei	Liver stages	UIS4 (PVM)	(Atchou, Berger et al. 2023, Calla, Mittal et al. 2023)
	Asexual blood stages	Tubulin, PolyE (microtubules)	(Bertiaux, Balestra et al. 2021, Ambekar 2022, Brusini, Dos Santos Pacheco et al. 2022, Rashpa, Klages et al. 2023)
		Centrin, FBXO1 (MTOC)	(Bertiaux, Balestra et al. 2021, Ambekar 2022, Brusini, Dos Santos Pacheco et al. 2022, Rashpa, Klages et al. 2023)
		NUF2, AKiT1 (Kinetochore)	(Brusini, Dos Santos Pacheco et al. 2022)
		Nup138, Nup313, Nup434 (Nuclear pore complex)	(Ambekar 2022)
	Ookinetes	Tubulin, PolyE, γ-tubulin (microtubules)	(Bertiaux, Balestra et al. 2021, Brusini, Dos Santos Pacheco et al. 2022, Dos Santos Pacheco, Brusini et al. 2022, Zeeshan, Rashpa et al. 2022, Rashpa, Klages et al. 2023)
		FXBO1, SKP1, CDPK1 (Pellicle)	(Rashpa, Klages et al. 2023)
		NUF2, SKA2 (Kinetochore)	(Brusini, Dos Santos Pacheco et al. 2022)
		SAS6-like, MyoB, PCR4, PCR5, GAC (APR)	(Bertiaux, Balestra et al. 2021, Dos Santos Pacheco, Brusini et al. 2022)
	Sporozoites	TRAP, CLAMP, AMA1 (micronemes)	(Loubens, Marinach et al. 2023)
	Gametocytes/ gametes	Tubulin, PolyE, γ-tubulin, kinesin-13 (microtubules)	(Bertiaux, Balestra et al. 2021, Brusini, Dos Santos Pacheco et al. 2022, Rashpa and Brochet 2022, Zeeshan, Rashpa et al. 2022, Rashpa, Klages et al. 2023)
		AKiT1, NUF2 (Kinetochore)	(Brusini, Dos Santos Pacheco et al. 2022)
		Centrin, SAS4, SAS6 (MTOC)	(Brusini, Dos Santos Pacheco et al. 2022, Rashpa and Brochet 2022, Rashpa, Klages et al. 2023)
		FXBO1, SKP1, FBXL2 (Microtubules/MTOC)	(Rashpa, Klages et al. 2023)
		GEX1 (nuclear envelope)	(Rashpa and Brochet 2022)
		Nup138 (Nuclear pore complex)	(Ambekar 2022)

581 **Table 1: Summary of all proteins localised by U-ExM using antibodies or tagged cell lines in *Plasmodium*.**

Organism	Lifecycle stage	Antibodies and tagged proteins (subcellular location)	References
Toxoplasma gondii	Tachyzoites	Tubulin, AcTub, PolyE, TLAP2 (microtubules)	(Pavlou, Touquet et al. 2020, Tosetti, Dos Santos Pacheco et al. 2020, Lentini, Ben Chaabene et al. 2021, Pacheco, Tosetti et al. 2021, Brusini, Dos Santos Pacheco et al. 2022, Dave, LaFavers et al. 2022, Dos Santos Pacheco, Brusini et al. 2022, Louvel, Haase et al. 2022, Montano, Anandkrishnan et al. 2022, Severo, Souza et al. 2022, Sparvoli, Delabre et al. 2022, Tomasina, Gonzalez et al. 2022, Yang, Doud et al. 2023)
		GAP45, MyoA, IMC3 & 10 (inner membrane complex)	(Pavlou, Touquet et al. 2020, Oliveira Souza, Jacobs et al. 2022)
		NUF2, SKA1, SKA2, AKiT1, CENP-C (kinetochore)	(Brusini, Dos Santos Pacheco et al. 2022, Tomasina, Gonzalez et al. 2022)
		Chromo1 (centromere)	(Brusini, Dos Santos Pacheco et al. 2022)
		Centrin1, EB1, Cep250L1 (MTOC)	(Brusini, Dos Santos Pacheco et al. 2022, Tomasina, Gonzalez et al. 2022)
		RNG2 (APRs)	(Tosetti, Dos Santos Pacheco et al. 2020)
		CRMPa & b, AC2,8,9 & 10 (Apex)	(Tosetti, Dos Santos Pacheco et al. 2020, Pacheco, Tosetti et al. 2021, Sparvoli, Delabre et al. 2022)
		FRM1, AKMT, GAC, PCR1,4,5,6 & 7, PPKL (PCRs)	(Dos Santos Pacheco, Brusini et al. 2022, Yang, Doud et al. 2023)
		Centrin 2 (Apical annuli/PCRs)	(Tosetti, Dos Santos Pacheco et al. 2020, Dos Santos Pacheco, Brusini et al. 2022)
		MyoH, AKMT, SAS6L, DCX, CPH1 (Conoid)	(Dos Santos Pacheco, Brusini et al. 2022, Louvel, Haase et al. 2022)
		RON2,3,4,9,13 (Rhoptries)	(Lentini, Ben Chaabene et al. 2021, Pacheco, Tosetti et al. 2021)
		EFP1 (Golgi/Plant-like vacuole)	(Dave, LaFavers et al. 2022)
		LMF1 (Mitochondrion)	(Oliveira Souza, Jacobs et al. 2022)
		Cpn60 (Apicoplast)	(Tomasina, Gonzalez et al. 2022)
		Actin	(Dos Santos Pacheco, Brusini et al. 2022)
		Histone H1-like (chromatin)	(Severo, Souza et al. 2022)
		MORN, PPKL (Basal complex)	(Tomasina, Gonzalez et al. 2022, Yang, Doud et al. 2023)

582 **Table 2: Summary of all proteins localised by U-ExM using antibodies or tagged cell lines in *Toxoplasma*.**

583

Organism	Lifecycle stage	Antibodies and tagged proteins (subcellular location)	References
Cryptosporidium parvum	Sporozoites	CB1 (Crystalloid body)	(Guérin, Strelau et al. 2023)
		ROP7 (Rhoptry)	(Guérin, Strelau et al. 2023)
		MIC2 (micronemes)	(Guérin, Strelau et al. 2023)
		DG1,2,3 & 4 (Dense granules)	(Guérin, Strelau et al. 2023)
		SG1 & 2 (Small granules)	(Guérin, Strelau et al. 2023)
		TSP1 (apical vesicles)	(John, M. Bader et al. 2023)
	Meront	DG2 (Dense granules)	(Guérin, Strelau et al. 2023)

584 **Table 3: Summary of all proteins localised by U-ExM using antibodies or tagged cell lines in *Cryptosporidium*.**

585

ACKNOWLEDGEMENTS

We thank Amandine Guérin (University of Geneva) and Boris Striepen (University of Pennsylvania) who contributed images *Cryptosporidium* images to this review, Virginie Hamel, Mathieu Brochet and Paul Guichard (University of Geneva), who contributed *Plasmodium* ookinete images to this review, and Rodolpho Ornitz Oliveira Souza and Gustavo Arrizabalaga (Indiana University School of Medicine) who contributed *Toxoplasma* images to this review. We thank James Blauwkamp (Indiana University School of Medicine) for feedback on the figures and tables in this review. We thank Maria Mota, Inês Bento, Nuno Leal (IMM, Lisboa), Alexis Kaushansky, Elizabeth Glennon, and Veronica Primavera (Seattle Children's Research Institute) for the samples the liver-stage, oocyst and sporozoite images were taken from. We thank Amandine Guérin (University of Geneva) for critical reading of the *Cryptosporidium* sections of the manuscript. We thank Gautam Dey (EMBL) and Omaya Dudin (EPFL) for sharing details of about the TREC ExM project.

COMPETING INTERESTS

The authors declare no competing interests or conflicts of interest.

AUTHOR CONTRIBUTIONS

Benjamin Liffner: Conceptualization, Investigation, Writing – Original Draft, Writing – Review & Editing.

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