Trends in
Biochemical Sciences

CellPress
OPEN ACCESS

Special Issue: Pushing boundaries of cryo-EM

Review

Structural parasitology of the malaria parasite *Plasmodium falciparum*

Leonie Anton,[1] David W. Cobb,[1] and Chi-Min Ho[1,*]

The difficulty of faithfully recapitulating malarial protein complexes in heterologous expression systems has long impeded structural study for much of the *Plasmodium falciparum* proteome. However, recent advances in single-particle cryo electron microscopy (cryoEM) now enable structure determination at atomic resolution with significantly reduced requirements for both sample quantity and purity. Combined with recent developments in gene editing, these advances open the door to structure determination and structural proteomics of macromolecular complexes enriched directly from *P. falciparum* parasites. Furthermore, the combination of cryoEM with the rapidly emerging use of *in situ* cryo electron tomography (cryoET) to directly visualize ultrastructures and protein complexes in the native cellular context will yield exciting new insights into the molecular machinery underpinning malaria parasite biology and pathogenesis.

Highlights

With half the world's population currently at risk, malaria remains a significant global health burden.

The difficulty of expressing many malarial protein complexes in heterologous systems has precluded structural and biochemical studies, impeding efforts to elucidate the functions and molecular mechanisms of many important but poorly understood biological pathways, including potential therapeutic targets.

Recent and ongoing advances in structure determination of macromolecular complexes using cryo electron microscopy (cryoEM) provide new avenues for structural study of the *P. falciparum* proteome, much of which previously resisted structure determination.

CryoEM of endogenously derived macromolecular complexes, enabled by the significantly reduced sample requirements of cryoEM studies, will lead to the discovery of so far unknown native substrates, binding partners, and modifications.

The malaria parasite *Plasmodium falciparum* poses specific challenges to structural studies

Malaria (see Glossary) exacts a devastating toll on global public health, with an estimated 3.8 billion people, half the world's population, currently at risk [1]. The recent rise of drug-resistant malaria parasites poses an urgent need to identify new targets for the development of antimalarial treatments with novel modes of action [2–7]. Furthermore, a large number of the biological pathways driving parasite biology and pathogenesis remain enigmatic. For instance, the molecular mechanisms by which parasite **effector proteins** are trafficked to key subcellular compartments such as the host cell membrane are largely unknown. The limited insight into these mechanisms hinders understanding of malaria parasite biology and impacts efforts to combat the pathogen.

Structure determination plays an important role in addressing these unknowns, often revealing previously unidentified interactions and pathways, and subsequently providing key insights into both the functions and molecular mechanisms of potential therapeutic targets. Unfortunately, there are several difficulties in recapitulating the proper folding and assembly of malarial protein complexes in heterologous systems that have precluded structural and biochemical study of many important *P. falciparum* protein complexes using conventional approaches. For example, the *P. falciparum* genome is exceptionally AT rich, with an average AT content of 80.6% and a heavily skewed **codon usage bias**, making cloning into heterologous expression systems challenging [8,9]. Although this challenge is now mitigated with the use of codon optimization algorithms [10], much of the proteome is highly aggregation-prone, littered with low-complexity regions and extensive charged-residue repeats, posing a major obstacle to heterologous expression of many *P. falciparum* proteins [11,12].

As evidenced by the severe paucity of high-resolution *P. falciparum* structures in the Protein Data Bank (PDB)[i] relative to other organisms, these obstacles have hampered structural studies of the

[1]Department of Microbiology and Immunology, Columbia University Irving Medical Center, New York, NY, USA

*Correspondence:
chi-min.ho@columbia.edu (C.-M. Ho).

P. falciparum proteome using conventional methods such as X-ray crystallography (XRC) and nuclear magnetic resonance (NMR) which depend heavily on the production of large amounts of highly purified protein via **recombinant overexpression** [13–16] (discussed further below). These challenges have hindered efforts to build a better understanding of novel malaria parasite biology. Consequently, many of the molecular mechanisms underlying the ability of the parasite to hijack human erythrocytes have remained enigmatic (Box 1).

Fortunately, the explosion of recent and ongoing advances in determining the structures [17–33] and dynamics [34–38] of macromolecular complexes using cryoEM have set in motion a paradigm shift in structural biology. This shift has particularly significant implications for the structural study of previously intractable biological systems such as *P. falciparum*, as there are no known homologs outside the *Plasmodium* genus for up to one-third of the proteome [39], and only 5% of the proteome has been structurally characterized[i]. In comparison, high-resolution structural information is available for more than 23% of the human proteome[i]. In this review we first discuss how cryoEM has enabled structure determination of previously intractable, recombinantly expressed *P. falciparum* proteins. We then highlight how several key advantages of the cryoEM technique have been leveraged to enable structure determination of endogenously expressed protein complexes enriched directly from native malaria parasites. Looking forward, we envisage an integrated view of malarial protein complexes in their native cellular contexts by combining high-resolution single-particle cryoEM structures with *in situ* **cryo electron tomography (cryoET)** imaging of protein complexes at subnanometer resolution in intact parasites.

CryoEM enables structure determination of previously intractable *P. falciparum* protein complexes overexpressed in recombinant systems

CryoEM is now routinely used to determine high-resolution structures of challenging proteins and complexes that were refractory to structure determination using XRC because they could only be produced in small quantities, even when recombinantly overexpressed [40]. Indeed, the amounts of protein required to achieve an atomic-resolution structure using cryoEM can be up to 3–4 orders of magnitude less than those typically required for XRC. Recently, cryoEM coupled with heterologous expression has enabled structure determination of key *P. falciparum* protein complexes that play important roles in antimalarial drug resistance and parasite invasion (Box 1).

For instance, a 3.2 Å single-particle cryoEM structure of the *P. falciparum* chloroquine resistance transporter (*Pf*CRT), a drug/metabolite transporter [41] that is responsible for conferring resistance to the antimalarial drugs chloroquine and piperaquine [42,43], was determined using *Pf*CRT expressed recombinantly in HEK293 cells and bound to a *Pf*CRT-specific antigen-binding fragment to overcome current cryoEM size limitations [44]. The structure reveals a cluster of mutations in the central cavity that transports physiological substrates across the digestive vacuole (DV) membrane (Figure 1A and Box 1). These mutations are known to affect the binding and export of chloroquine or piperaquine from the DV by *Pf*CRT, and the study provides a structural framework for understanding the molecular basis of parasite resistance to these drugs [44].

In addition, two recent cryoEM studies of proteins on the **merozoite** surface have helped to expand our understanding of merozoite invasion [45–47] (Box 1). The family of merozoite surface proteins (MSPs) form a fibrillar coat on the surface of the parasite and play roles in invasion. The most abundant MSP – *Pf*MSP1 – binds the erythrocyte surface proteins glycophorin A and band 3, and is crucial for successful invasion [48–51]. CryoEM was used to determine a 3.1 Å structure of recombinantly expressed *Pf*MSP1 monomer (Figure 1B), as well as two homodimeric forms and five other subtly different monomeric confirmations, highlighting the flexibility of the *Pf*MSP1 structure [45]. *Pf*MSP1 interacts with many other MSPs and erythrocyte proteins, and

Glossary

Amphipathic: a molecule that has both hydrophilic and hydrophobic properties.
Apicomplexa: a phylum of unicellular parasites.
Apicoplast: an organelle found in most apicomplexan parasites that has four membranes and is the site of many metabolic pathways.
Codon usage bias: the use of specific codons during mRNA translation versus other synonymous codons.
Cryo-electron tomography (cryoET): a technique in which a series of 2D images, known as a tilt series, is collected from a sample as it is tilted.
Cryo-focused ion beam (cryo-FIB) milling: the process of ablating frozen cell material using the ion beam in a cryo-FIB scanning electron microscope (cryo-FIB-SEM) for the purpose of creating a thin cross-section of the cell for cryoET imaging.
Effector proteins: proteins of a pathogen that modulate the function and metabolism of the host cell.
Immunogenic epitopes: molecular patterns of a pathogen that can elicit an immune response.
Lamella: a thin cross-section of a frozen cell created in a cryo-FIB-SEM for cryoET imaging.
Malaria: a disease caused by the pathogen *Plasmodium* that is characterized by intense cyclic fevers.
Merozoite: the invasive, asexual form of *Plasmodium* in the blood stage that infects erythrocytes.
Micrograph: an image taken using a microscope.
Recombinant or heterologous expression: expression of protein(s) in model organisms such as *Escherichia coli*, *Saccharomyces cerevisiae*, insect, or mammalian cell lines rather than in the source organism, often at much higher expression levels (known as overexpression).
Sporozoites: these develop from sexual reproduction in the mosquito midgut and are injected into a human host during a mosquito blood-feed.
Structural proteomics: uses structure determination to identify specific proteins in a complex mixture of proteins.
Tilt series: a series of 2D images taken of an object or area of interest from a range of angles during cryoET data collection.
Tomogram: a 3D reconstruction of a slice or section through a 3D object, reconstructed from a tilt series.

Box 1. Malaria life cycle and erythrocyte invasion

Malaria is a disease caused by single-cell, eukaryotic parasites from the genus *Plasmodium*. Of the human-infecting species of *Plasmodium*, *P. falciparum* is associated with the most severe forms of the disease and the highest mortality. The definitive host of *P. falciparum* is the *Anopheles* mosquito which transmits parasites to the human host during a blood feed. Within a newly infected human, parasites traverse to the liver, where they rapidly replicate before egressing into the bloodstream to initiate the asexual replication cycle that occurs within erythrocytes (red blood cells, RBCs; Figure I). All the clinical manifestations of malaria are associated with the asexual, intraerythrocytic replication of the parasites, highlighting the need to study the asexual lifecycle. Within the erythrocyte, the parasite feeds on host cell hemoglobin to support its own growth before replicating into 16–32 daughter parasites, known as merozoites (Figure IA). The merozoites egress from the erythrocyte, remaining briefly extracellular while merozoite surface proteins interact with receptors on a new erythrocyte host (Figure IB). After finding its new host cell, the merozoite reorients such that its apical end is in contact with the erythrocyte, and then the parasite forces itself into the host cell (Figure IC). After entering the erythrocyte, the cycle begins again: the parasite exports hundreds of proteins into the host to create its niche (Figure ID) and imports hemoglobin to consume and prepare for a new round of replication. A subset of asexual parasites exit this replication cycle to develop into gametocytes that are taken up into a new mosquito host for sexual development and propagation to a new human host.

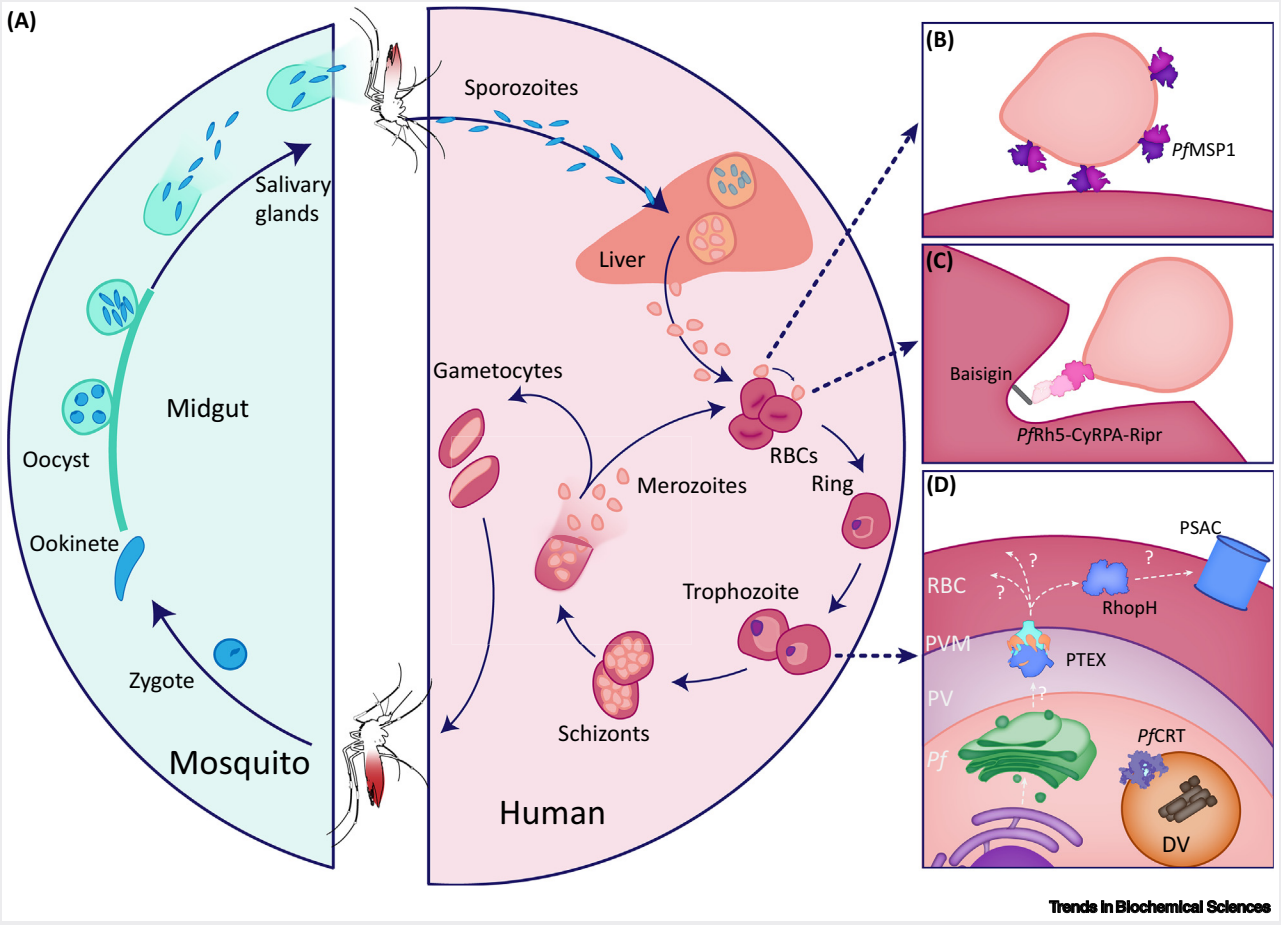

Figure I. Malaria life cycle and erythrocyte invasion. Abbreviations: DV, digestive vacuole; PV, parasitophorous vacuole; PVM, PV membrane; PTEX, *Plasmodium* translocon of exported proteins; RBCs, red blood cells; PSAC, plasmodial surface anion channel .

its inherent flexibility may underlie the diversity of these interactions [48–50,52–56]. Importantly, the cryoEM structure enabled mapping of **immunogenic epitopes** [57–59] for *Pf*MSP1, which is a candidate for vaccine development.

Following initial interaction with the erythrocyte, the binding of surface-exposed reticulocyte-binding protein homolog 5 (*Pf*Rh5), in complex with the proteins cysteine-rich protective antigen

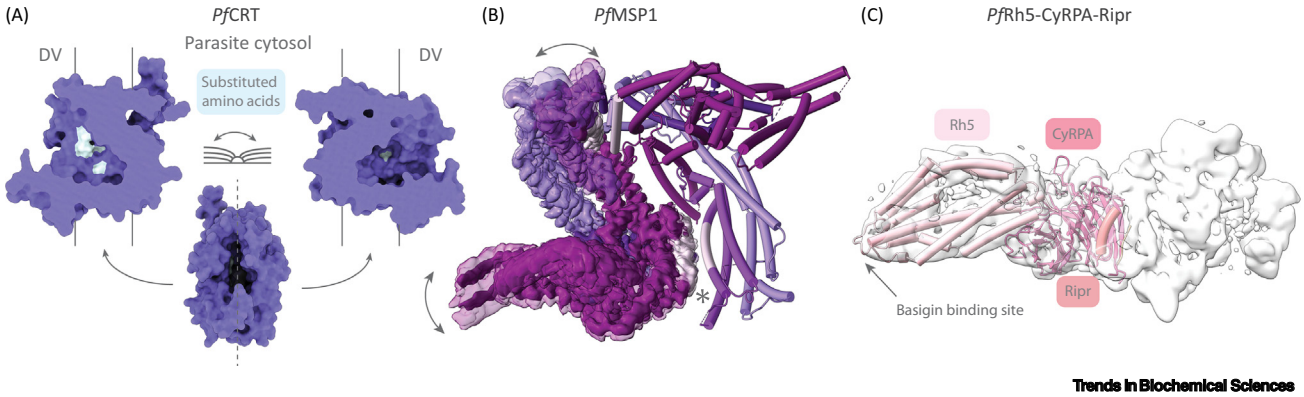

Figure 1. *Plasmodium falciparum* cryo electron microscopy (cryoEM) protein structures from recombinant expression systems. (A) Surface representation of *Pf*CRT cryoEM structure (PDB ID: 6UKJ)[i] [44]. Substituted amino acids in cavity, resulting from genetic mutations that enable chloroquine (CQ) and piperaquine (PPQ) binding, are colored light blue. (B) CryoEM structure of the *Pf*MSP1 dimer [45]. The left protomer shows reconstruction of the six different monomer conformations found in sample, with arrows indicating flexibility (EMD-11150-55)[ii]. The right protomer is shown as a cylindrical representation of the atomic model (PDB ID: 6ZBJ)[i]. The asterisk indicates the interface between two protomers which is highlighted in light pink. (C) CryoEM reconstruction of the Rh5–CyRPA–Ripr–basigin complex (EMD-9192)[ii] [46]. Cylindrical representation for Rh5 (light pink), CyRPA (coral), and Ripr (salmon); the basigin binding site is indicated with an arrow (PDB ID: 6MPV)[i]. Representations for all reconstructions and models were created in ChimeraX [101]. Abbreviations: CRT, chloroquine resistance transporter; DV, digestive vacuole; MSP, merozoite surface protein; Rh5, reticulocyte-binding protein homolog 5; Ripr, Rh5-interacting protein.

(*Pf*CyRPA) and Rh5-interacting protein (*Pf*Ripr), to the erythrocyte membrane protein basigin is required for successful invasion [54,60,61]. Furthermore, the complex is likely involved in the formation of a pore between parasite and host, enabling an influx of Ca^{2+} ions that is essential to complete invasion into the erythrocyte [61–64]. Despite its importance for invasion, the structure of the ternary complex remained unknown until the recent publication of two cryoEM structures of recombinantly expressed *Pf*CyRPA–Ripr and *Pf*Rh5–CyRPA–Ripr complexes at overall resolutions of 5.07 Å and 7.17 Å, respectively [46] (Figure 1C). Importantly, the ternary structure revealed that the three subunits assemble in a 1:1:1 ratio, with *Pf*CyRPA acting as a core that joins *Pf*Rh5 and *Pf*Ripr. Fitting a previously published *Pf*Rh5–basigin crystal structure [65] into the ternary complex cryoEM density yielded insights into the possible mechanism of *Pf*Rh5–Ripr insertion into the erythrocyte membrane; briefly, the N-terminus of *Pf*Rh5 binds to basigin, orienting the *Pf*Rh5–Ripr complex parallel to the erythrocyte membrane. This positions the **amphipathic** *Pf*Rh5 C-terminal helical bundle close to the membrane, potentially initiating insertion into the membrane. The cryoEM structure of the ternary complex provides an important step forward in understanding the role of PfRh5–CyRPA–Ripr in parasite invasion and will enable future studies seeking to block invasion by interfering with the complex.

CryoEM enables structure determination of endogenous *P. falciparum* protein complexes

In the previously described studies, the reduced sample requirement associated with cryoEM was leveraged to enable structure determination of noteworthy protein complexes that eluded characterization by XRC, leading to important observations about the biology of the parasite. However, another implication of the reduced sample requirement should not be overlooked: the requirement is sufficiently low to enable cryoEM structure determination of endogenously derived complexes [66–70]. In addition, unlike XRC, which requires highly purified samples to allow billions of identical protein molecules to pack together into a highly ordered crystal lattice, protein molecules in cryoEM samples are preserved in a frozen-hydrated state [71,72], thereby capturing the full range of conformations and orientations sampled by the molecules while in aqueous solution. The ramifications for *P. falciparum* and other non-model organisms with proteomes that resist expression in recombinant systems are significant. CryoEM structure

determination of endogenous protein complexes enriched directly from the parasite presents an exciting path forward for the many challenging protein complexes that are not amenable to expression in heterologous systems, enabling structure determination of these important complexes in near-native states. Further, this approach ensures the presence of biologically relevant post-translational modifications and potentially allows the identification of previously undiscovered native substrates or binding partners, and even the discovery and characterization of previously unknown protein complexes.

CRISPR-assisted tagging of endogenous proteins enables structure determination of complexes enriched directly from *P. falciparum* parasites using cryoEM

Combined with advances in CRISPR/Cas9-assisted parasite gene editing that enable the addition of affinity tags to proteins of interest for purification [69,73–75], the endogenous approach to cryoEM structure determination can be quite powerful. For example, this approach was first used to determine the structure and mechanism of an essential malarial membrane protein complex known as the *Plasmodium* translocon of exported proteins (PTEX) [69]. PTEX is the sole gateway for export of hundreds of **effector proteins** into the host cell, a process essential to the ability of the parasite to inhabit and reproduce within human erythrocytes (Box 1). To obtain the long-sought structure of this novel translocon, endogenous PTEX was enriched directly from *P. falciparum* parasites via an epitope tag inserted using CRISPR/Cas9 technology into the genetic locus encoding a PTEX subunit. Single-particle cryoEM was then used to determine structures of the native PTEX complex in two distinct functional states, yielding the first reported near-atomic-resolution cryoEM structures of protein(s) isolated directly from a native source using an epitope tag inserted into the endogenous locus with CRISPR/Cas9 gene editing [69].

Remarkably, native cargo protein peptides were observed still bound in the central channel of the PTEX translocon in both structures (Figure 2), a direct consequence of the fact that the PTEX complex was enriched from parasites harvested at a point in the life cycle when they are actively exporting hundreds of effector proteins [69]. This serendipitous outcome provided a key insight into the

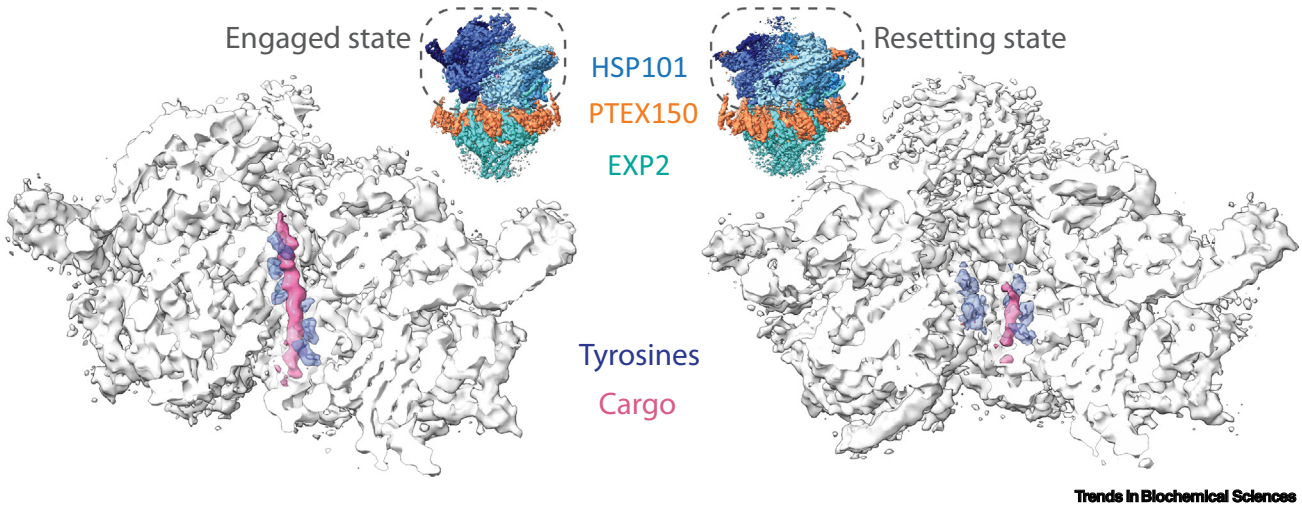

Figure 2. Cryo electron microscopy (cryoEM) of the endogenous *Plasmodium falciparum PTEX* complex reveals native cargo. CryoEM density maps of HSP101 from the PTEX translocon in transparent white with cargo (pink) and interdigitating pore loop tyrosines (purple, stick representation) in the engaged and resetting states (EMD-8951, EMD-8952)[ii]. Maps are bisected to show the endogenous cargo and pore loop tyrosines in the protein-unfolding channel of the HSP101 unfoldase. Comparing the positioning of these pore loops relative to the cargo between the two states suggests a model for the mechanisms by which HSP101 unfolds the cargo protein and threads it through the transmembrane channel of the translocon. Full PTEX complex structures are shown in the insets for context (PDB IDs: 6E10, 6E11)[i]. Abbreviations: EXP2, exported protein component 2; HSP101, malaria heat shock protein 101; PTEX, *Plasmodium* translocon of exported proteins.

molecular mechanism of effector protein translocation by the PTEX translocon (Figure 2) that would have been missed had the complex been artificially reconstituted in a heterologous system.

This work demonstrates that, by enabling structure determination of endogenously derived samples, cryoEM not only provides a path forward for many previously intractable malarial macromolecular complexes but also presents the possibility of observing as yet undiscovered native substrates, binding partners, or post-translational modifications [76], none of which would be found in samples produced in heterologous systems. Similar unanticipated observations have been reported in a growing number of endogenous structures [69,76–81], and it is increasingly clear that the potential for discovering native binding partners, substrates, or modifications represents a major advantage of endogenous cryoEM that should be capitalized on.

Leveraging cryoEM to discover previously unknown protein complexes within heterogeneous samples enriched from *P. falciparum* cell lysates

With cryoEM, protein molecules are more likely to be captured in near-native and biologically relevant states, and the resulting 2D projections of individual protein molecules in a dataset of cryoEM **micrographs**, known as particles (Figure 3A), can be extensively classified before distinct subsets of self-similar particles are averaged to yield the final high-resolution 3D reconstructions (Figure 3B,C) [24,26,32,82,83]. During these powerful 2D and 3D classification steps, the sample is subjected to what is in essence an additional *in silico* purification step. Because of this extra step, sample heterogeneity that would be prohibitive for XRC is not only tolerated but can sometimes be leveraged to achieve multiple high-resolution structures of a single protein complex in different conformational states (Figure 3D) [69,84,85], or even several distinct structures of completely unrelated protein complexes from a single cryoEM dataset [68].

We recently developed a cryoEM-enabled endogenous **structural proteomics** approach that leverages the capacity for *in silico* purification to determine multiple structures from coarsely fractionated parasite lysates containing a heterogeneous mixture of protein complexes (Figure 4A, key figure) [68]. Parasite lysates are subjected to sucrose gradient fractionation, and the protein complexes in the resulting fractions are identified using a combination of cryoEM, mass spectrometry, and cryoID, a program that identifies proteins in cryoEM density maps of unknown protein complexes at better than 4.0 Å, without prior knowledge of their primary sequence(s) (Figure 4A–C) [68]. This approach was used to identify and determine the structures of three *P. falciparum* protein complexes –

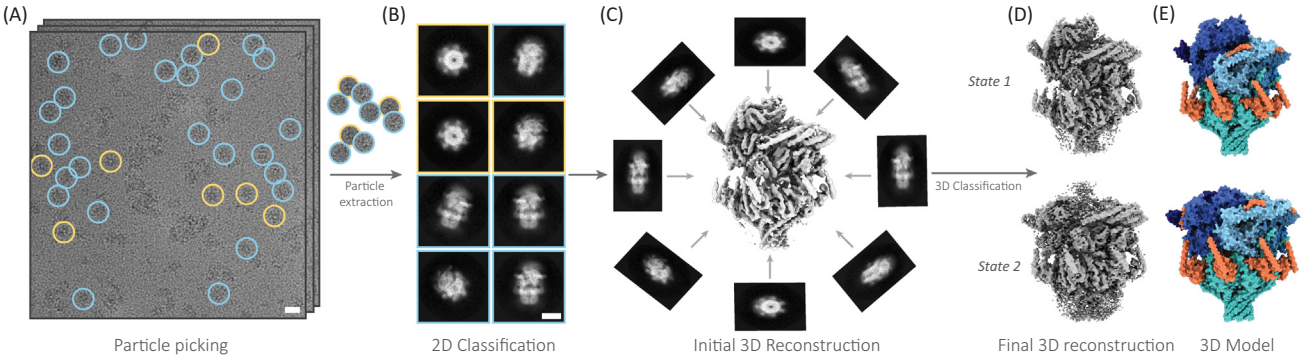

Figure 3. Single-particle cryo electron microscopy (cryoEM) data-processing workflow. (A) CryoEM micrographs of a protein sample. Particles are identified, or 'picked', then extracted from the micrographs. (B) Extracted particles are sorted into classes containing self-similar views, and are then averaged to produce 2D class averages. (C) Particles from high-resolution 2D class averages are then used to calculate 3D reconstructions. (D) 3D reconstructions are further classified and refined to yield one or more final high-resolution cryoEM density maps, which enable atomic model building (E).

Key figure

Cryo electron microscopy (cryoEM), focused ion beam scanning electron microscopy (FIB-SEM), and *in situ* cryo electron tomography (cryoET) workflow

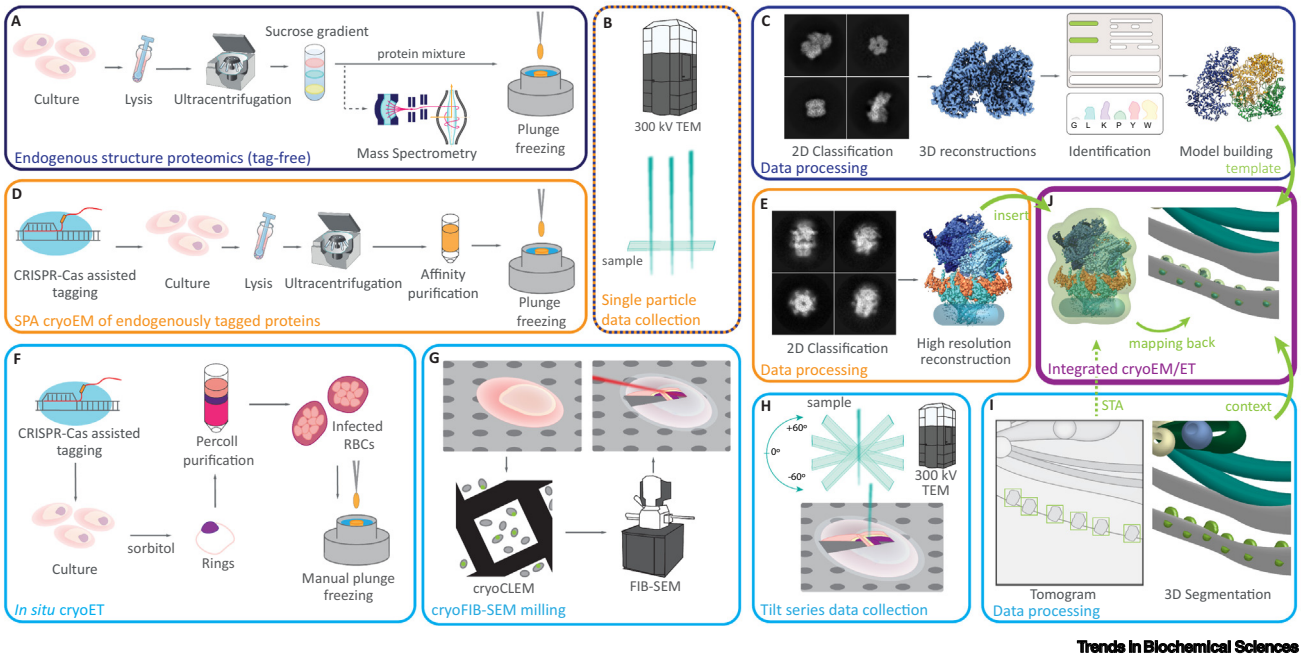

Figure 4. (A) Tag-free sample preparation for endogenous structural proteomics. Protein complexes are enriched from malaria parasite lysates using sucrose gradient fractionation. Mass spectrometry and negative-stain electron microscopy are used to identify fractions containing protein complexes of interest, which are then plunge-frozen on cryoEM grids for (B) single-particle cryoEM imaging in a high-resolution 300 kV transmission electron microscope (TEM). (C) CryoEM analysis yields near-atomic-resolution cryoEM density maps. CryoID software is used to identify the protein(s) in the maps, enabling model building of atomic-resolution structures. (D) Sample preparation for endogenous CRISPR-tagged single-particle cryoEM. Affinity tags are inserted into the endogenous loci of proteins of interest in malaria parasites using CRISPR/Cas9 gene editing. Tagged proteins are affinity purified from parasite lysates, plunge-frozen on cryoEM grids, and used for single-particle cryoEM imaging (B). (E) CryoEM analysis yields near-atomic-resolution cryoEM density maps, enabling atomic model building. (F) Sample preparation for *in situ* cryoET. Proteins of interest are fluorescently tagged using CRISPR/Cas9 gene editing. The resulting transgenic *P. falciparum* parasites are grown in synchronous culture, and parasite-infected red blood cells (RBCs) are isolated and plunge-frozen directly on cryoEM grids. (G) Vitrified RBCs are then thinned in a dual-beam cryo-focused ion beam scanning electron microscope (cryo-FIB-SEM), yielding sections 100–200 nm in thickness called lamellae. Vitrified grids can be visualized in a cryo-correlative light and electron microscope (cryo-CLEM) to identify promising sites for cryo-FIB milling. (H) Tilt series are collected on lamella using a dose-symmetric tilt scheme ranging from –60° to +60° on a 300 kV TEM. (I) Tilt series are aligned and reconstructed into 3D volumes called tomograms. 3D segmentation and subtomogram averaging (STA) are then used to reveal subcellular details at subnanometer resolutions. (J) Integrating atomic-resolution information from single-particle cryoEM with the cellular context from *in situ* cryoET provides further insights into the molecular mechanisms underlying parasite biology and pathogenesis. High-resolution reconstructions from cryoEM can be inserted into lower-resolution subtomogram averages to give context on the immediate environment. Reconstructions can also be mapped back to the original 3D segmentation for cellular context.

glutamine synthetase, M18 aspartyl aminopeptidase, and the 20S proteasome – from images of a single sucrose-gradient fraction in a proof-of-principle study [68]. To further demonstrate the power of this approach, the endogenous structural proteomics approach was used in a second study to determine a structure of the *P. falciparum* RhopH complex [70].

The endogenous structural proteomics approach will be particularly impactful in organisms such as *P. falciparum* where dissection of important protein–protein interactions is often laborious owing to the genetic intractability of the parasite.

In situ cryoET holds potential to resolve cellular ultrastructures of intact, parasite-infected erythrocytes at subnanometer resolution

The single-particle cryoEM studies described thus far have all made significant contributions to our understanding of various aspects of malaria parasite biology and pathogenesis. However, one major drawback of single-particle cryoEM studies is their dependence on purified protein complexes. During the purification process, red blood cells and their resident parasites must be broken open, destroying the cellular context within which macromolecular complexes exist, along with many important clues to the function and mechanism of the proteins in their native environments.

The loss of cellular context can be addressed using cryoET, a technique wherein a series of images are taken of a vitrified, intact cell from many angles, and are then aligned and combined to produce a 3D reconstruction of the cell, called a **tomogram**, to directly visualize intracellular features within their preserved context. For example, earlier this year, a cryoET study of the *Trypanosoma brucei* flagellum revealed the architecture underlying the flagellum-driven, nonplanar helical motility of the parasite, thereby elucidating how the specific manner in which the axoneme and the paraflagellar rod are connected enables the typical movement of *T. brucei* that is essential for infection [86]. In **apicomplexan** parasites, cryoET has been used to investigate the apex of *Toxoplasma* parasites [87], the membrane structure of the *Plasmodium* **apicoplast** [88], and the nuclear pore complexes and microtubule structures within *Plasmodium* **sporozoites** [89,90].

However, sample thickness is one of the major determinants of achievable resolution in cryoET and imposes a limit on the types of samples that can be investigated with this method. Consequently, cryoET studies such as those listed above have been limited to analyses of isolated organelles, the thinnest areas of cells like the parasite apex, or life stages that are naturally thinner, for example *Plasmodium* sporozoites.

The recent development of **cryo-focused ion beam (cryo-FIB) milling**, that uses a cryo-FIB-scanning electron microscope (cryo-FIB-SEM) to create thin 100–200 nm sections, called **lamellae**, of vitrified, unfixed cells has enabled cryoET beyond naturally thin cell areas [91]. This combination of cryo-FIB milling with cryoET, known as *in situ* cryoET (Figure 4F–H), enables the collection of **tilt series** anywhere in any cell, and with significantly improved contrast, thus yielding tomographic reconstructions in which supramolecular features and even individual protein complexes are resolved at unprecedented subnanometer resolution [92,93]. Further averaging of repeated particles within a tomogram using a method called subtomogram averaging can yield 3D reconstructions of the particles at resolutions approaching sub-4 Å [28]. These reconstructions can then be mapped back into the full cellular tomogram to capture the cellular context (Figure 4I) [94–96].

Although the potential advances enabled by *in situ* cryoET are significant, it is still a relatively new technique that is subject to a fair share of limitations and challenges (see Outstanding questions). Even with recent innovations in automation, cryo-FIB milling remains a laborious, time-consuming, and low-throughput technique. The achievable resolution for subtomogram averaging depends heavily on the quality of the sample and the number of protein complexes that can be extracted from a tomogram [97]. Ribosomes and proteasomes are highly abundant in cells and can therefore be found and targeted with relative ease, as can repetitive ultrastructures. However, many proteins of interest are more difficult to pinpoint in a crowded cellular environment, and targeting specific areas of the cell remains challenging. Using cryo-correlative light electron microscopy (cryo-CLEM), light microscopy images of cells expressing fluorescently tagged proteins can be mapped onto SEM images of the same sample, enabling reasonable targeting of specific regions in the *x–y* direction for FIB milling [98].

However, for accurate mapping in the *z* direction, specialized confocal [99] or super-resolution microscopes [98,100] are needed in combination with correlative software programs. To avoid ice contamination from transferring devices and to improve ease of correlation, high-resolution light objectives and detectors that can be integrated into the cryo-FIB-SEM instrument are currently under development, but the resolution in *z* remains a significant obstacle. Despite current limitations, *in situ* cryoET provides a window into cells at an unprecedented resolution.

Concluding remarks

The use of cryoEM is quickly advancing our understanding of malaria parasite structural biology and of the mechanisms underpinning *P. falciparum* invasion and subjugation of human erythrocytes. By combining the subnanometer to near-atomic-resolution cellular context attainable using *in situ* cryoET and subtomogram averaging with atomic-resolution structural information from single-particle cryoEM (Figure 4J) (see Outstanding questions), we will be able to directly visualize the molecular machinery underlying novel parasite biology and host–pathogen interactions at atomic resolution. These high-resolution insights into *P. falciparum* biology will be invaluable tools in the continuous need to develop new strategies for combating the parasite.

Outstanding questions

The rapidly evolving repertoire of single-particle cryoEM tools is making structural biology increasingly accessible to the previously disparate cell biology community. However, significant barriers to entry remain for *in situ* cryoET, even for those within the cryoEM community. What key innovations would help to alleviate this problem?

What additional steps can the cryoEM community take to remove barriers to entry to structural biology for the cell biology community, and for the parasitology community in particular?

How can transient interactions and biological processes such as those mediating invasion and exflagellation during the *P. falciparum* life cycle be addressed with cryoEM and *in situ* cryoET?

How can cryoEM and *in situ* cryoET be leveraged for structural study of malaria parasites in the liver and vector-borne stages of the life cycle?

Acknowledgments

We thank Michael Reese, Vasant Muralidharan, and Anthony W.P. Fitzpatrick for helpful comments regarding the manuscript. We regret that the diversity of the topics discussed in this review prevented us from being able to cite many more excellent papers. C-M.H. is supported by a National Institutes of Health (NIH) Director's Early Independence Award (DP5OD029613) and a Precision Medicine Pilot Grant from the Columbia Precision Medicine Initiative. L.A. is supported by a Postdoctoral Mobility Fellowship from the Swiss National Science Foundation (200205).

Declaration of interests

The authors declare no conflicts of interest.

Resources

[i]Protein Data Bank (PBD): https://www.rcsb.org/

[ii]Electron Microcopy Data Bank (EMBD) at the European Bioinformatics Institute: https://www.ebi.ac.uk/emdb/

References

1. World Health Organization (2018) *World Malaria Report 2018*, WHO, p. 210
2. Uwimana, A. *et al.* (2020) Emergence and clonal expansion of in vitro artemisinin-resistant *Plasmodium falciparum* kelch13 R561H mutant parasites in Rwanda. *Nat. Med.* 26, 1602–1608
3. Mathieu, L.C. *et al.* (2020) Local emergence in Amazonia of *Plasmodium falciparum* k13 C580Y mutants associated with in vitro artemisinin resistance. *Elife* 9, e51015
4. Conrad, M.D. and Rosenthal, P.J. (2019) Antimalarial drug resistance in Africa: the calm before the storm? *Lancet Infect. Dis.* 19, e338–e351
5. Menard, D. *et al.* (2018) Multidrug-resistant *Plasmodium falciparum* malaria in the Greater Mekong subregion. *Lancet Infect. Dis.* 18, 238–239
6. Thu, A.M. *et al.* (2017) Combating multidrug-resistant *Plasmodium falciparum* malaria. *FEBS J.* 284, 2569–2578
7. Menard, D. and Fidock, D.A. (2019) Accelerated evolution and spread of multidrug-resistant *Plasmodium falciparum* takes down the latest first-line antimalarial drug in southeast Asia. *Lancet Infect. Dis.* 19, 916–917
8. Carlton, J.M. *et al.* (2008) Comparative genomics of the neglected human malaria parasite *Plasmodium vivax*. *Nature* 455, 757–763
9. Weber, J.L. (1987) Analysis of sequences from the extremely A+T-rich genome of *Plasmodium falciparum*. *Gene* 52, 103–109
10. Burgess-Brown, N.A. *et al.* (2008) Codon optimization can improve expression of human genes in *Escherichia coli*: a multi-gene study. *Protein Expr. Purif.* 59, 94–102
11. Aravind, L. *et al.* (2003) *Plasmodium* biology: genomic gleanings. *Cell* 115, 771–785
12. Muralidharan, V. and Goldberg, D.E. (2013) Asparagine repeats in *Plasmodium falciparum* proteins: good for nothing? *PLoS Pathog.* 9, e1003488
13. Derewenda, Z.S. (2004) The use of recombinant methods and molecular engineering in protein crystallization. *Methods* 34, 354–363
14. Hopp, T.P. *et al.* (1988) A short polypeptide marker sequence useful for recombinant protein identification and purification. *Bio-Technology* 6, 1204–1210
15. Porath, J. *et al.* (1975) Metal chelate affinity chromatography, a new approach to protein fractionation. *Nature* 258, 598–599
16. Rosenberg, A.H. *et al.* (1987) Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene* 56, 125–135
17. Cheng, Y. (2015) Single-particle cryo-EM at crystallographic resolution. *Cell* 161, 450–457
18. Li, X. *et al.* (2013) Electron counting and beam-induced motion correction enable near-atomic-resolution single-particle cryo-EM. *Nat. Methods* 10, 584–590
19. Liu, H. *et al.* (2010) Atomic structure of human adenovirus by cryo-EM reveals interactions among protein networks. *Science* 329, 1038–1043
20. McMullan, G. *et al.* (2009) Detective quantum efficiency of electron area detectors in electron microscopy. *Ultramicroscopy* 109, 1126–1143
21. McMullan, G. *et al.* (2009) Enhanced imaging in low dose electron microscopy using electron counting. *Ultramicroscopy* 109, 1411–1416

22. Zhang, X. *et al.* (2010) 3.3 A cryo-EM structure of a nonenveloped virus reveals a priming mechanism for cell entry. *Cell* 141, 472–482
23. Bai, X.C. *et al.* (2015) An atomic structure of human gamma-secretase. *Nature* 525, 212–217
24. Punjani, A. *et al.* (2017) CryoSPARC: algorithms for rapid unsupervised cryo-EM structure determination. *Nat. Methods* 14, 290–296
25. Rohou, A. and Grigorieff, N. (2015) CTFFIND4: fast and accurate defocus estimation from electron micrographs. *J. Struct. Biol.* 192, 216–221
26. Scheres, S.H.W. (2012) RELION: implementation of a Bayesian approach to cryo-EM structure determination. *J. Struct. Biol.* 180, 519–530
27. Tegunov, D. and Cramer, P. (2019) Real-time cryo-electron microscopy data preprocessing with Warp. *Nat. Methods* 16, 1146–1152
28. Tegunov, D. *et al.* (2021) Multi-particle cryo-EM refinement with M visualizes ribosome-antibiotic complex at 3.5 A in cells. *Nat. Methods* 18, 186–193
29. Zhang, K. (2016) Gctf: real-time CTF determination and correction. *J. Struct. Biol.* 193, 1–12
30. Zhang, K. (2019) Gautomatch: a GPU-accelerated program for accurate, fast, flexible and fully automatic particle picking from cryo-EM micrographs with or without templates. *GitHub* Published online March 16, 2019. https://github.com/scipion-em/scipion-em-gautomatch/wiki/ProtGautomatch
31. Zheng, S.Q. *et al.* (2017) MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy. *Nat. Methods* 14, 331–332
32. Grant, T. *et al.* (2018) cisTEM, user-friendly software for single-particle image processing. *Elife* 7, e35383
33. Liao, M. *et al.* (2013) Structure of the TRPV1 ion channel determined by electron cryo-microscopy. *Nature* 504, 107–112
34. Punjani, A. and Fleet, D.J. (2021) 3D variability analysis: resolving continuous flexibility and discrete heterogeneity from single particle cryo-EM. *J. Struct. Biol.* 213, 107702
35. Punjani, A. *et al.* (2020) Non-uniform refinement: adaptive regularization improves single-particle cryo-EM reconstruction. *Nat. Methods* 17, 1214–1221
36. Nakane, T. and Scheres, S.H.W. (2021) Multi-body refinement of cryo-EM images in RELION. *Methods Mol. Biol.* 2215, 145–160
37. Zhong, E.D. *et al.* (2021) CryoDRGN: reconstruction of heterogeneous cryo-EM structures using neural networks. *Nat. Methods* 18, 176–185
38. Chen, M. and Ludtke, S.J. (2021) Deep learning-based mixed-dimensional Gaussian mixture model for characterizing variability in cryo-EM. *Nat. Methods* 18, 930–936
39. Böhme, U. *et al.* (2019) Progression of the canonical reference malaria parasite genome from 2002–2019. *Wellcome Open Res.* 4, 58
40. Nogales, E. (2016) The development of cryo-EM into a mainstream structural biology technique. *Nat. Methods* 13, 24–27
41. Martin, R.E. and Kirk, K. (2004) The malaria parasite's chloroquine resistance transporter is a member of the drug/metabolite transporter superfamily. *Mol. Biol. Evol.* 21, 1938–1949
42. Dhingra, S.K. *et al.* (2017) A variant PfCRT isoform can contribute to *Plasmodium falciparum* resistance to the first-line partner drug piperaquine. *mBio* 8, e00303-17
43. Fidock, D.A. *et al.* (2000) Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Mol. Cell* 6, 861–871
44. Kim, J. *et al.* (2019) Structure and drug resistance of the *Plasmodium falciparum* transporter PfCRT. *Nature* 576, 315–320
45. Dijkman, P.M. *et al.* (2021) Structure of the merozoite surface protein 1 from *Plasmodium falciparum*. *Sci. Adv.* 7, eabg0465
46. Wong, W. *et al.* (2019) Structure of *Plasmodium falciparum* Rh5–CyRPA–Ripr invasion complex. *Nature* 565, 118–121
47. Cowman, A.F. *et al.* (2017) The molecular basis of erythrocyte invasion by malaria parasites. *Cell Host Microbe* 22, 232–245
48. Baldwin, M.R. *et al.* (2015) Merozoite surface protein 1 recognition of host glycophorin A mediates malaria parasite invasion of red blood cells. *Blood* 125, 2704–2711
49. Boyle, M.J. *et al.* (2010) Interactions with heparin-like molecules during erythrocyte invasion by *Plasmodium falciparum* merozoites. *Blood* 115, 4559–4568
50. Das, S. *et al.* (2015) Processing of *Plasmodium falciparum* merozoite surface protein MSP1 activates a spectrin-binding function enabling parasite egress from RBCs. *Cell Host Microbe* 18, 433–444
51. O'Donnell, R.A. *et al.* (2000) Functional conservation of the malaria vaccine antigen MSP-119across distantly related *Plasmodium* species. *Nat. Med.* 6, 91–95
52. Kauth, C.W. *et al.* (2006) Interactions between merozoite surface proteins 1, 6, and 7 of the malaria parasite *Plasmodium falciparum*. *J. Biol. Chem.* 281, 31517–31527
53. Lin, C.S. *et al.* (2016) Multiple *Plasmodium falciparum* merozoite surface protein 1 complexes mediate merozoite binding to human erythrocytes. *J. Biol. Chem.* 291, 7703–7715
54. Wright, K.E. *et al.* (2014) Structure of malaria invasion protein RH5 with erythrocyte basigin and blocking antibodies. *Nature* 515, 427–430
55. Paul, G. *et al.* (2018) Protein–protein interaction studies reveal the *Plasmodium falciparum* merozoite surface protein-1 region involved in a complex formation that binds to human erythrocytes. *Biochem. J.* 475, 1197–1209
56. Ranjan, R. *et al.* (2011) Proteome analysis reveals a large merozoite surface protein-1 associated complex on the *Plasmodium falciparum* merozoite surface. *J. Proteome Res.* 10, 680–691
57. Blank, A. *et al.* (2020) Immunization with full-length *Plasmodium falciparum* merozoite surface protein 1 is safe and elicits functional cytophilic antibodies in a randomized first-in-human trial. *NPJ Vaccines* 5, 10
58. Fowkes, F.J. *et al.* (2010) The relationship between anti-merozoite antibodies and incidence of *Plasmodium falciparum* malaria: a systematic review and meta-analysis. *PLoS Med.* 7, e1000218
59. Osier, F.H. *et al.* (2008) Breadth and magnitude of antibody responses to multiple *Plasmodium falciparum* merozoite antigens are associated with protection from clinical malaria. *Infect. Immun.* 76, 2240–2248
60. Crosnier, C. *et al.* (2011) Basigin is a receptor essential for erythrocyte invasion by *Plasmodium falciparum*. *Nature* 480, 534–537
61. Volz, J.C. *et al.* (2016) Essential role of the PfRh5/PfRipr/CyRPA complex during *Plasmodium falciparum* invasion of erythrocytes. *Cell Host Microbe* 20, 60–71
62. Chen, L. *et al.* (2011) An EGF-like protein forms a complex with PfRh5 and is required for invasion of human erythrocytes by *Plasmodium falciparum*. *PLoS Pathog.* 7, e1002199
63. Reddy, K.S. *et al.* (2015) Multiprotein complex between the GPI-anchored CyRPA with PfRH5 and PfRipr is crucial for *Plasmodium falciparum* erythrocyte invasion. *Proc. Natl. Acad. Sci. U. S. A.* 112, 1179–1184
64. Weiss, G.E. *et al.* (2015) Revealing the sequence and resulting cellular morphology of receptor-ligand interactions during *Plasmodium falciparum* invasion of erythrocytes. *PLoS Pathog.* 11, e1004670
65. Chen, L. *et al.* (2014) Crystal structure of PfRh5, an essential *P. falciparum* ligand for invasion of human erythrocytes. *eLife* 3, e04187
66. Wong, W. *et al.* (2014) Cryo-EM structure of the *Plasmodium falciparum* 80S ribosome bound to the anti-protozoan drug emetine. *eLife* 3, e03080
67. Li, H. *et al.* (2016) The cryo-EM structure of the *Plasmodium falciparum* 20S proteasome and its use in the fight against malaria. *FEBS J.* 283, 4238–4243
68. Ho, C.M. *et al.* (2020) Bottom-up structural proteomics: cryoEM of protein complexes enriched from the cellular milieu. *Nat. Methods* 17, 79–85
69. Ho, C.M. *et al.* (2018) Malaria parasite translocon structure and mechanism of effector export. *Nature* 561, 70–75
70. Ho, C.M. *et al.* (2021) Native structure of the RhopH complex, a key determinant of malaria parasite nutrient acquisition. *Proc. Natl. Acad. Sci. U. S. A.* 118, e2100514118
71. Dubochet, J. and McDowall, A.W. (1981) Vitrification of pure water for electron microscopy. *J. Microsc.* 124, 3–4

72. Dubochet, J. *et al.* (1982) Electron microscopy of frozen water and aqueous solutions. *J. Microsc.* 128, 219–237
73. Ghorbal, M. *et al.* (2014) Genome editing in the human malaria parasite *Plasmodium falciparum* using the CRISPR-Cas9 system. *Nat. Biotechnol.* 32, 819–821
74. Wagner, J.C. *et al.* (2014) Efficient CRISPR-Cas9-mediated genome editing in *Plasmodium falciparum*. *Nat. Methods* 11, 915–918
75. Schureck, M.A. *et al.* (2021) Malaria parasites use a soluble RhopH complex for erythrocyte invasion and an integral form for nutrient uptake. *eLife* 10, e65282
76. Poweleit, N. *et al.* (2016) CryoEM structure of the *Methanospirillum hungatei archaellum* reveals structural features distinct from the bacterial flagellum and type IV pilus. *Nat. Microbiol.* 2, 16222
77. Maxson, M.E. *et al.* (2021) Detection and quantification of the vacuolar H^+-ATPase using the *Legionella* effector protein SidK. *BioRxiv* Published online July 30, 2021. https://doi.org/10.1101/2021.07.29.454369
78. Herbst, D.A. *et al.* (2021) Structure of the human SAGA coactivator complex: the divergent architecture of human SAGA allows modular coordination of transcription activation and cotranscriptional splicing. *BioRxiv* Published online February 8, 2021. https://doi.org/10.1101/2021.02.08.430339
79. Dai, D.L. *et al.* (2021) Structural characterization of endogenous tuberous sclerosis protein complex revealed potential polymeric assembly. *Biochemistry* 60, 1808–1821
80. Puchades, C. *et al.* (2017) Structure of the mitochondrial inner membrane AAA+ protease YME1 gives insight into substrate processing. *Science* 358, eaao0464
81. Mashtalir, N. *et al.* (2020) A structural model of the endogenous human BAF complex informs disease mechanisms. *Cell* 183, 802–817
82. Scheres, S.H.W. (2012) A Bayesian view on Cryo-EM structure determination. *J. Mol. Biol.* 415, 406–418
83. Tang, G. *et al.* (2007) EMAN2: an extensible image processing suite for electron microscopy. *J. Struct. Biol.* 157, 38–46
84. Hofmann, S. *et al.* (2019) Conformation space of a heterodimeric ABC exporter under turnover conditions. *Nature* 571, 580–583
85. Zhu, Y. *et al.* (2018) Structural mechanism for nucleotide-driven remodeling of the AAA-ATPase unfoldase in the activated human 26S proteasome. *Nat. Commun.* 9, 1360
86. Zhang, J. *et al.* (2021) Structure of the trypanosome paraflagellar rod and insights into non-planar motility of eukaryotic cells. *Cell Discov.* 7, 51
87. Aquilini, E. *et al.* (2021) An Alveolata secretory machinery adapted to parasite host cell invasion. *Nat. Microbiol.* 6, 425–434
88. Lemgruber, L. *et al.* (2013) Cryo-electron tomography reveals four-membrane architecture of the *Plasmodium* apicoplast. *Malar. J.* 12, 25
89. Kehrer, J. *et al.* (2018) Nuclear pore complex components in the malaria parasite *Plasmodium berghei*. *Sci. Rep.* 8, 11249
90. Kudryashev, M. *et al.* (2012) Structural basis for chirality and directional motility of *Plasmodium* sporozoites. *Cell. Microbiol.* 14, 1757–1768
91. Wagner, F.R. *et al.* (2020) Preparing samples from whole cells using focused-ion-beam milling for cryo-electron tomography. *Nat. Protoc.* 15, 2041–2070
92. Marko, M. *et al.* (2007) Focused-ion-beam thinning of frozen-hydrated biological specimens for cryo-electron microscopy. *Nat. Methods* 4, 215–217
93. Mahamid, J. *et al.* (2016) Visualizing the molecular sociology at the HeLa cell nuclear periphery. *Science* 351, 969–972
94. Guo, Q. *et al.* (2018) In situ structure of neuronal C9orf72 poly-GA aggregates reveals proteasome recruitment. *Cell* 172, 696–705
95. Albert, S. *et al.* (2017) Proteasomes tether to two distinct sites at the nuclear pore complex. *Proc. Natl. Acad. Sci. U. S. A.* 114, 13726–13731
96. Albert, S. *et al.* (2020) Direct visualization of degradation microcompartments at the ER membrane. *Proc. Natl. Acad. Sci. U. S. A.* 117, 1069–1080
97. Zhang, P. (2019) Advances in cryo-electron tomography and subtomogram averaging and classification. *Curr. Opin. Struct. Biol.* 58, 249–258
98. Tuijtel, M.W. *et al.* (2019) Correlative cryo super-resolution light and electron microscopy on mammalian cells using fluorescent proteins. *Sci. Rep.* 9, 1369
99. Arnold, J. *et al.* (2016) Site-specific cryo-focused ion beam sample preparation guided by 3D correlative microscopy. *Biophys. J.* 110, 860–869
100. Hoffman, D.P. *et al.* (2020) Correlative three-dimensional super-resolution and block-face electron microscopy of whole vitreously frozen cells. *Science* 367, eaaz5357
101. Pettersen, E.F. *et al.* (2021) UCSF ChimeraX: structure visualization for researchers, educators, and developers. *Protein Sci.* 30, 70–82