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REVIEW ARTICLE

Ultrastructure expansion microscopy: Enlarging our perspective on apicomplexan cell division

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Abstract

Apicomplexans, a large phylum of protozoan intracellular parasites, well known for their ability to invade and proliferate within host cells, cause diseases with major health and economic impacts worldwide. These parasites are responsible for conditions such as malaria, cryptosporidiosis, and toxoplasmosis, which affect humans and other animals. Apicomplexans exhibit complex life cycles, marked by diverse modes of cell division, which are closely associated with their pathogenesis. All the unique structural and evolutionary characteristics of apicomplexan parasites, the biology underlying life stage transitions, and the singular mechanisms of cell division alongside their associated biomedical relevance have captured the attention of parasitologists of all times. Traditional light and electron microscopy have set the fundamental foundations of our understanding of these parasites, including the distinction among their modes of cell division. This has been more recently complemented by microscopy advances through the implementation of superresolution fluorescence microscopy, and variants of electron microscopy, such as cryo-EM and tomography, revealing intricate details of organelles and cell division. Ultrastructure Expansion Microscopy has emerged as a transformative, accessible approach that enhances resolution by physically expanding samples isometrically, allowing nanoscale visualisation on standard light microscopes. In this work, we review the most recent contributions of U-ExM and its recent improvements and innovations, in providing unprecedented insights into apicomplexan ultrastructure and its associated mechanisms, focusing particularly on cell division. We highlight the power of U-ExM in combination with protein-specific labelling, in aiding the visualisation of long oversighted organelles and detailed insights into the assembly of parasite-specific structures, such as the conoid in *Plasmodia*, and the apical-basal axis in *Toxoplasma*, respectively, during new parasite assembly. Altogether, the contributions of U-ExM reveal conserved and unique structural features across species while nearing super resolution. The development of these methodologies and their combination with different technologies are crucial for advancing our mechanistic understanding of apicomplexan biology,

offering new perspectives that may facilitate novel therapeutic strategies against apicomplexan-caused diseases.

KEYWORDS
apicomplexan cell division, endodyogeny, endopolygeny, schizogony, ultrastructure expansion microscopy

1 | INTRODUCTION

Apicomplexans constitute a large phylum of eukaryotic intracellular parasites known for their ability to invade and replicate within the cells of their hosts. Apicomplexans have long captivated scientists' attention as they are not only notable causative agents of many global neglected diseases, but they are also notable examples of evolution and adaptation. Apicomplexans cause deadly diseases disproportionately impacting children, such as malaria, and cryptosporidiosis, as well as the widely prevalent toxoplasmosis, among others. Apicomplexan-caused diseases affect humans and other animals, underlying considerable health and economic burdens.[1] The phylum takes its name after its so-called apical complex which derives from the Latin: *apex* (top) and *complexus* (complex). The apical complex is composed of a sophisticated assembly of cytoskeletal structures and secretory organelles, including tubulin fibres that conform the conoid, working in synergy with the rhoptries, micronemes, and dense granules. Together these elements form a perfect injection weapon which is crucial for host cell invasion and intracellular survival. An additional notable feature of apicomplexans is that they evolved from two endosymbiotic events that gave rise to two membranous organelles; a one per cell mitochondria and apicoplast.[2,3] Both the mitochondria and the apicoplast are present in most, but not all, members of this phylum, as some members, such as the *Cryptosporidia* have experienced organelle secondary losses.[4]

Apicomplexan also rely on a range of microtubule-based cytoskeletal structures during their life-cycle stages.[5] One of them is the previously mentioned apical complex, which together with the cortical microtubules are held in place by an apical Microtubule Organizing Center (MTOC), known as the apical polar ring or APR. Notably, apicomplexans house a second MTOC, which coordinates cell division through the nucleation of the mitotic spindle, and controlling organelle positioning.[6]

Despite sharing these broadly conserved features, apicomplexans exhibit significant biological diversity, including variations in host species and target cell types.[7] Diversity extends to their complex life cycles and unique mechanisms of cell division. Cell division underlies the capacity of apicomplexans to fill and eventually lyse their host cells, a mechanism of pathogenesis tightly linked to the clinical manifestations of apicomplexan-caused diseases. Understanding these modes of division, in the context of their life cycles, is crucial for developing effective strategies to combat the diseases they cause.[8]

Invariably, all life cycles involve invading host cells. However, depending on the invaded host, species within the phylum can undergo asexual or sexual replication, prior to transmission to a new host.[8–12] In addition, a single species can showcase different cell division modes depending on its life stage. Nonetheless, cell division invariably happens through unique mechanisms which arise from combinations of closed nuclear mitosis and de novo daughter cell assembly, varying at the level of how these two processes are temporally and spatially coordinated. For example, during *schizogony* of the malaria causing agent *Plasmodium*, DNA duplication and karyokinesis occur simultaneously and repeatedly, generating a syncytium which can generate tenths to hundreds of daughter cells upon daughter cell assembly at the surface concomitantly with the last round of nuclear mitosis. Endodyogeny in *Toxoplasma gondii* (causative of toxoplasmosis) entails sequential DNA duplication and karyokinesis, the latter accompanied by the internal formation of two daughter cells. Endopolygeny with karyokinesis, as showcased by, for example, *Cystoisospora suis* (causative agent of cystoisosporiasis) generates, similarly to schizogony, a syncytium, but which will form daughters internally. Finally, species dividing by endopolygeny without karyokinesis, such as the case of *Sarcocystis* (agent of sarcocystosis), generate a large polyploid nucleus prior to internal daughter assembly. Note that these modes of division have been extensively revised recently.[8] We succinctly summarise them in Figure 1 for reference throughout this work.

Many efforts have pursued and greatly advanced the elucidation of the molecular architecture of apicomplexan parasites.[13–15] Until recently, our fundamental understanding of cell cycle and cell division had been largely shaped by the work of electron microscopists, which were fundamentally insightful in terms of molecular architecture. However, the molecular understanding of the cell cycle progression and life stage conversion

in apicomplexans,[16,17] such as *Toxoplasma gondii*[18,19] and *Plasmodium*,[20,21] have significantly advanced in recent years. This progress is largely due to technological advancements in genetic manipulation, but also in microscopy, with the development of technologies that better capture dynamic processes, allowing temporal resolution upon the implementation of live imaging, and enhanced imaging processivity accompanied by advanced image analysis. This combination has provided deeper insights into the processes underlying the apicomplexan's complex life cycles.[8,22–24] These innovations have enhanced our ability to study the intricate mechanisms governing parasite differentiation, replication, and adaptation across species and different stages of infection.

Practitioner points

Apicomplexans divide by divergent cell division modes whose mechanisms can be exploited for new drug target discovery

Innovations in Ultrastructure Expansion Microscopy (U-ExM) provide amped resolution of minute structures

U-ExM has contributed to further our understanding of the cellular biology underlying Apicomplexan proliferation

2 | TECHNOLOGICAL INNOVATION IN MICROSCOPY APPLIED TO APICOMPLEXANS

The study of cell biology in apicomplexan parasites is complicated by their small size, ranging from 1 to 15 μm, a challenge similarly faced by researchers working on organisms of akin dimensions (bacteria, yeast, etc.). This has historically demanded the application of high-resolution microscopy and has therefore entailed that cutting-edge microscopy techniques have been applied promptly after surging to investigate the tiny internal structure and fundamental processes undergone by apicomplexans.

Notably, the advent of superresolution has played an important role in elucidating the detailed distribution of proteins within and among organelles uncovering uncharacterised compartments and functional compartmentalisation. Stimulated Emission Depletion (STED) allows researchers to observe structures and molecular processes at the nanoscale. This has improved our understanding of organelle dynamics, apical complex formation, and parasite invasion mechanisms.[25] For example, the application of dual colour STED uncovered that the once regarded as cytosolic centriolar plaque (a functionally homologous structure to the more widely conserved eukaryotic centrosome) in malaria parasites is indeed intranuclear and is located within a chromatin-free region.[26] Some variations of the technique, such as RescueSTED (REduction of State transition Cycles) nanoscopy, facilitated the study of microtubules and nuclear pores and allowed the characterisation of detailed organisation throughout the *Plasmodium* blood stage cycle.[27]

3D-structured illumination microscopy (3D-SIM) enhances image resolution by computationally reconstructing the finer details that are encoded for in an interference pattern.[28,29] The advent of 3D-SIM and its application in apicomplexan parasites was fundamental for uncovering the dual compartment organisation of the centrosome in *T. gondii*.[24]

The possibility of imaging live cells by time-lapse microscopy, confocal and light-sheet microscopy, has enabled the observation of apicomplexan parasites in real time, giving insights into how these parasites undergo cell division, replication, stage differentiation, and movement.[30,31]

The implementation of Fluorescence Lifetime Imaging Microscopy (FLIM) allows the study of intracellular environments by measuring the lifetime of fluorescent proteins. Metabolic changes that occur during stage conversion can be measured, such as those implied in the transition among life forms in apicomplexans. For example, *T. gondii* infection alters host cell metabolism, impacting processes such as redox balance and the binding activities of reduced NADH, NADPH, and FAD. Using noninvasive auto-fluorescence based lifetime imaging of single cells over the course of infection, along with specific metabolite analysis, extracellular flux analysis, and monitoring of reactive oxygen species (ROS) production, it is possible to study *T. gondii* infection consequences on the host cell. The autofluorescence and lifetime differences between the reduced forms of NAD and FAD in living cells allow the measurement of changes in the redox balance within the cell. This combined approach allowed for detailed insights into the metabolic shifts that occur in host cells during infection.[32]

Multiphoton Microscopy, useful for deep-tissue imaging, has been particularly beneficial for studying parasites in their natural environments, within host cells or tissues. It has helped understanding host-parasite interactions and, for example, the cellular niches in which persistence and stage conversion occur.[33] Two-photon microscopy contributed to understanding how CD8(+) T cell responses are initiated during infection with *T. gondii*.[34] It has also contributed to observing the dynamics of T cell–parasite interactions within living tissue providing new

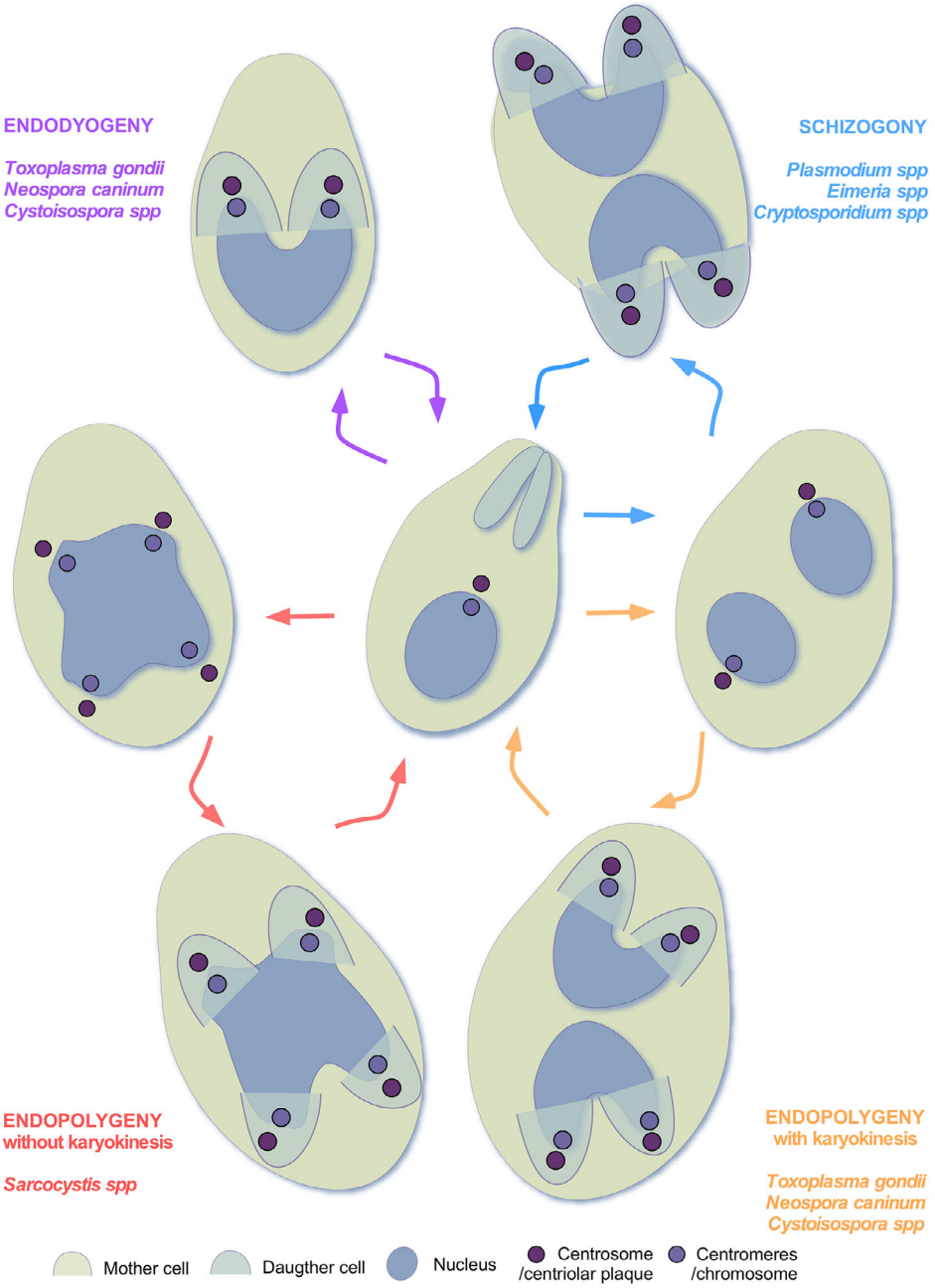

FIGURE 1 **Schematic representation of the cell division modes used by apicomplexa parasites**. Parasite genus using the different cell division modes are indicated. Note that the colour codes used in the arrows correspond to the cell division modes specified in Table 1.

perspectives for understanding immune responses to pathogens that are able to persist in the brain.[35]

Cryo-electron microscopy (Cryo-EM) has provided high-resolution structural details of parasite organelles, such as the apicoplast and the intricate organisation of the membranous sheets that underlie the cortical microtubules, critical for parasite proliferation and motility. Moreover, Cryo-EM, in combination with electron tomography, have been instrumental in elucidating the three-dimensional structure of complex cellular machinery, including complexes critical for rhoptry secretion[36] and mitochondrial cristae architecture,[28,29,37] among many others.

While these advancements in microscopy and imaging techniques have significantly enhanced our understanding of apicomplexan biology, they also require access to highly specialised equipment and technology. This can be a limiting factor for many labs or institutions, especially those with underdeveloped infrastructure or scarce funding. On the other hand, large volumes of data are generated that

need to be processed and analysed whilst many of these technologies lack the processivity required for multi-image acquisition, required for quantitative analyses.

In this context, Expansion Microscopy (from here on referred to as ExM) has emerged as a technique that enhances the resolution of conventional light microscopy by physically expanding the biological sample isometrically, allowing for maintenance of relative distances within the sample, through a relatively accessible chemical treatment. ExM was first introduced by the team of Edward Boyden,[38] where Chen and colleagues demonstrated that by expanding biological samples anchored to a swellable polymer, imaging resolution could be significantly improved using standard optical microscopes. ExM protocols follow a common workflow to achieve isotropic specimen expansion while preserving nanoscopic biological information. First, biomolecules of interest are labelled with gel-anchorable fluorophores. Molecular handles are then attached to biomolecules or labels, enabling binding to a swellable hydrogel. This hydrogel, formed via free-radical polymerisation of sodium acrylate with a cross-linker, mechanically couples the biomolecules to the polymer mesh. The specimen is then homogenised through chemical or enzymatic treatments optimising mechanical properties. Finally, water is introduced, causing the hydrogel to swell through osmotic force. Postexpansion, additional labelling or amplification can enhance visualisation.[39] Implementation of the ExM protocol results in approximately 4.5-fold, this factor is set by the cross-linker concentration isotropic ultrastructural expansion of the sample, while preserving both cells and isolated organelles, and allowing the maintenance of their native architecture for detailed imaging.[40,41] The isotropic expansion usually is verified by measurement of internal subcellular structures of known size.[38] The use of ExM rapidly spread among biologists and was applied to different systems and biomolecules revealing intricate ultrastructural details that are critical for understanding dynamic cellular processes, which were previously underappreciated. Refinements of the ExM protocol have allowed nanoscale imaging of biological molecules other than proteins. For example, a protocol applicable to RNA (expansion fluorescence in situ hybridisation, or ExFISH,[38] allows RNA-linking to the polymer, followed by single molecule identification by hybridisation of RNA-specific fluorescently labelled oligonucleotide probes.

Though the success of ExM marked a clear milestone in the development of techniques to circumvent the diffraction limits of a conventional microscope, ExM relied heavily on custom made anchorable labels or linkers, raising a barrier for its widespread adoption. In this light, variants of ExM rapidly surged building onto the original ExMs' concept. Innovations into the ExM protocol for label retention have allowed for conservation and enhancement of ExM signals through the use of specific antibody-based probes (as for example, in Label retention-ExM; LR-ExM).[42] Likewise, the Tetrahedral DNA Nanostructure Expansion Microscopy (TDN-ExM) was developed to enhance fluorescence labelling through tetrahedral DNA nanostructures, achieving a 3- to 10-fold signal amplification.[43] These innovations, many of which stand out for using chemicals and instruments found in a typical biology laboratory, have been reviewed in detail by Asano et al.[44]

Protein retention ExM (proExM) surged as an alternative, in which proteins, rather than labels, are anchored to the swellable gel. Herein, genetically encoded fluorescent proteins, epitope tags, or streptavidin, sufficiently retain their structural integrity to allow antibody specific recognition postgel swelling.[45] More recently developed MAP (Magnified Analysis of Proteome) allows for expansion while preserving cell fluorescence.[46]

Together, innovations which conserve protein integrity, gave rise to U-ExM (Ultrastructure Expansion Microscopy). Unlike ExM, which focuses on expanding the fluorophore imprint, U-ExM allows the preservation of the proteome during the expansion process. Additionally, U-ExM allows for postexpansion labelling using commercially available standard antibodies making this an accessible technique, requiring only conventional fluorescence microscopes downstream of sample processing. Incorporation of fluorescently-conjugated N-hydroxysuccinimide (NHS) esters, which bind to free amine groups[47] has facilitated Pan-proteome labelling allowing for visualisation of ultrastructure without the need for specific antibody labelling. This innovation has been adopted not only for the labelling of isolated expanded cells, such as HeLa[48,49] and RPE,[50] but also for expanded tissues and whole-organisms, such as kidney[50] and *C. elegans*,[51] respectively.

U-ExM enables nanoscale imaging at resolutions as low as 50–70 nm using conventional light microscopy, outperforming the resolution obtained by structured illumination superresolution. The expansion protocol was further optimised to amp expansion to 10-fold implementing Ten-fold Robust Expansion (TREx)[52] or 25-fold using iterative U-ExM (iU-ExM).[53] These innovations which further increase the resolution of U-ExM, come close to the resolution of electron microscopy (EM), but without the need for the use of costly equipment or extensive technical expertise as that required for EM image acquisition. Unsurprisingly, U-ExM has become the go-to powerful and accessible tool for studying subcellular structures. In this work, we review the use of U-ExM for the study of cell division in apicomplexan parasites.

3 | ULTRASTRUCTURE OF DIVISION OF APICOMPLEXAN PARASITES USING EXPANSION MICROSCOPY

U-ExM has been widely incorporated and proven extremely powerful for studying cell biology of many unicellular organisms including *Trypanosma cruzi*,[54] *Trypanosoma brucei*,[55,56] *Chlamydomonas*,[57] and *Lecudina tuzetae* in detail.[58] In addition, protocols have been tailored and improved for use in a wide variety of apicomplexans.[20,53,58–64] NHS ester staining was used to visualise apical polar rings and other structures during the blood stages in *Plasmodium*. For this, NHS ester was combined with tubulin staining, highlighting these cytoskeletal features in two distinct expanded life stages (schizonts and merozoites) showcasing its utility for precise structural visualisation in this species.[60] This study led to the conclusion that a conoid-like structure is conserved in *Plasmodium* species, contesting a largely accepted paradigm that only coccidian species within the apicomplexan phylum displayed conservation of this structure.[60] More recently, NHS ester, in combination with the nuclear probe Sytox, was used by Liffner and colleagues to reconstruct a high-resolution protein map throughout asexual blood stage development of malaria parasites.[64]

Tosetti et al.[25] pioneered the use of U-ExM in *Toxoplasma* with great results, spearheading its use which quickly expanded to other apicomplexans. Detailed accounts of U-ExMs applications in *Toxoplasma* will be mentioned throughout this work. Recent innovations, such as iU-ExM have also been applied to apicomplexans. In *Toxoplasma gondii*, iU-ExM allowed for the visualisation of the eightfold symmetry of the inner segments of nuclear pores, as well as, the detailed organisation of microtubules within the conoid and the centriolar microtubules organisation. These structures had only been described in detail before by transmission electron microscopy.[53]

4 | EXPANSION MICROSCOPY AND ITS INSIGHTS INTO APICOMPLEXAN CELL DIVISION

Apicomplexans use a variety of divergent cell division strategies, which further vary along their differentiation into distinct life stages (Figure 1). The latter are tightly linked to their life cycles, while the former is optimised in a life-stage specific manner to ensure either rapid proliferation and successful transmission between hosts, or latency and persistence. Recently, the contributions of expansion microscopy to the studies of apicomplexan cell division in the best studied models within the phylum, *Plasmodium*, *Toxoplasma*, and *Cryptosporidium* were extensively reviewed by Liffner and Absalon.[65] Here, we will review the most recent lights shed onto these mechanisms of division as well as the stage-specific features revealed in detail by U-ExM both in model apicomplexans as well as in understudied members of the phylum. While we will focus on *Toxoplasma* and its endodyogeny, as most U-ExM studies have been performed in light of this cell division mode, we will also discuss the contribution of the implementation of this technique to less well understood division modes used by other apicomplexans (Table 1).

4.1 | Apicomplexan division by endodyogeny

Toxoplasma gondii is a promiscuous parasite in terms of its host range and cell type preferences. Its multiple infection routes, broad host cell range (virtually any warm-blooded species), capacity to invade almost any nucleated cell, and ability to persist chronically make it one of the most successful zoonotic parasites worldwide with a third of the world population being infected.[66] *Toxoplasma* has a fast-dividing form, known as the tachyzoite, responsible for acute infection (Figure 2A). The tachyzoite can be easily grown and maintained in vitro; hence, it is the best-studied *T. gondii* life stage. Tachyzoites divide via a process called endodyogeny, involving one round of DNA replication, closed nuclear mitosis, and the formation of two daughter cells within the mother cell[67] (Figure 2B–D). *Toxoplasma*, like most other Apicomplexans, relies on various tubulin-based structures, which include centrioles, conoid fibres, subpellicular and intraconoidal microtubules, and a cortical cytoskeleton, to progress through division. Invariably, daughter cells' cytoskeleton is assembled de novo, ensuring clear and immediate differentiation between the developing daughter cytoskeletons and the fully established maternal structure.[68] During de novo daughter cell formation two poles marked by the apical and basal complexes, respectively, are readily defined (Figure 2C and D). The basal complex plays a crucial role in the structure and division by capping and constricting the cell's posterior end. MORN1 is essential for maintaining the constriction at the posterior end of the parasite and is important for successful cytokinesis.[69,70] Though a great deal of detailed understanding of the events leading up to a successful endodyogeny has been attained, mechanistic details have suffered from lack of resolution within the minute structures that make up emerging daughter cells.

The implementation of U-ExM has recently allowed the detailed examination of the development of the apical complex (including the conoid and the apical microtubule

TABLE 1 Summary of most recent UEx-M contributions to structural insights within the different modes of cell division used by apicomplexans.

Division mode	Subcellular structure observed	Type of ExM	Expansion factor	References[a]
Endodyogeny	MTs, conoid	iU-ExM	16×	Haase et al., 2024[62]
	Apical complex	U-ExM	4×	Dos Santos Pacheco et al., 2021[77]
	MT nucleation	pan-ExM	13×	Engelberg et al., 2024[75]
	Conoid	iU-ExM	22.4×	Louvel et al., 2023[53]
	Cyst wall	StcE U-ExM	4.0×–4.3×	Bondarenko et al., 2024[91]
	Apical polar ring and the basal complex	U-ExM[b]	No data	Arias Padilla et al., 2024[71]
	Cortical MT and conoid	U-ExM[b]	5.4×	Arias Padilla, Murray et al., 2024[72]
	Mitotic spindle	U-ExM	3.5×	Tomasina et al., 2022[85]
	Mitochondria	U-ExM	4×	Oliveira Souza et al., 2022[87], 2024[88]
	Centrocone, basal complex	U-ExM	No data	Hawkins et al., 2024[81]
	Nucleus and nucleolus	U-ExM	No data	Severo et al., 2022[86]
	Alveolin network	U-ExM	4×	Tosetti et al., 2020[25]
Endopolygeny	Subpellicular microtubules	Pan-ExM	13×	Engelberg et al., 2024[75]
Schizogony	MT nucleation	iU-ExM	16×	Haase et al., 2024[62]
	Subpellicular MT, conoid	U-ExM	4×	Bertiaux et al., 2021[60]
	MTOC	pan-ExM	10×	Rashpa and Brochet, 2022[100]
	Host and parasite ultrastructure	MoTissU-ExM	4.36×	Liffner et al., 2024[20]
	Mitotic spindle	U-ExM	4.3×	Liffner and Absalon, 2021[63]
	Kinetochore	U-ExM	No data	Li et al., 2024[93]
	Secretory organelles-rhoptry	U-ExM	No data	Anaguano et al., 2024[94]
	Mitochondria	PS-ExM	5×	Atchou et al., 2023[59]
	Centrosome, apicoplast, Golgi, ER, secretory organelles	U-ExM	4.5×	Liffner and Absalon, 2023[65]
	Kinetochore/mitotic spindle	U-ExM	No data	Brusini et al., 2022[99]

[a]References listed include only the most recent work regarding ultrastructure expansion microscopy with emphasis in cell division. Note that previous references and their details were recently listed and revised by Liffner and Absalon.[65]

[b]Note that in these studies, U-ExM was coupled to structured illumination microscopy.

organising centre, the apical polar ring), the cortical MTs, and the basal complex.[71,72] This was made possible due to the increased imaging processivity of U-ExM, which allowed repeated observation of a rare stage of the cell cycle, which is difficult to find by EM. In addition, labelling microtubule-based structures together with a basal complex marker allowed isolated observation of the process along cell cycle progression. This study has uncovered that the primordial basal complex assembles in the earliest detectable daughter cell structures, while the near concentric arc of the primordial apical polar ring is assembled simultaneously. The temporal determination of these events together with the amped resolution given by U-ExM has provided unprecedented insight, allowing authors to postulate this as the mechanism for cell determination of the apical-basal axis. The understanding of basal complex formation and its role in daughter cell formation, has been aided by the identification by proteomics of basal end components, labelled collectively as 'BC' proteins. BCs identified by proteome identified a number of new basal complex components, which in early mitosis, orchestrate together with MORN1, the anchoring of microtubule ends and the growth of the forming daughter cells. This mechanistic insight was made possible by the combined use of a BCC4 mutant, together with U-ExM whereby discrete foci were resolved along the ring formed by MORN1 for BC proteins.[73]

A defining feature of apicomplexan parasites is their cytoskeleton, composed of the inner membrane complex (IMC) supported by intermediate-like filaments and subpellicular microtubules (SPMTs). In *Toxoplasma gondii*, this cytoskeleton plays a key role in the lytic cycle and serves as a scaffold for daughter cell assembly during budding. Using U-ExM in combination with 3D-SIM, Padilla and colleagues observed that the assembly of the microtubule scaffold, which eventually matures into a fully assembled daughter cell, is a step-wise process in which structures form hierarchically. Specifically, the formation

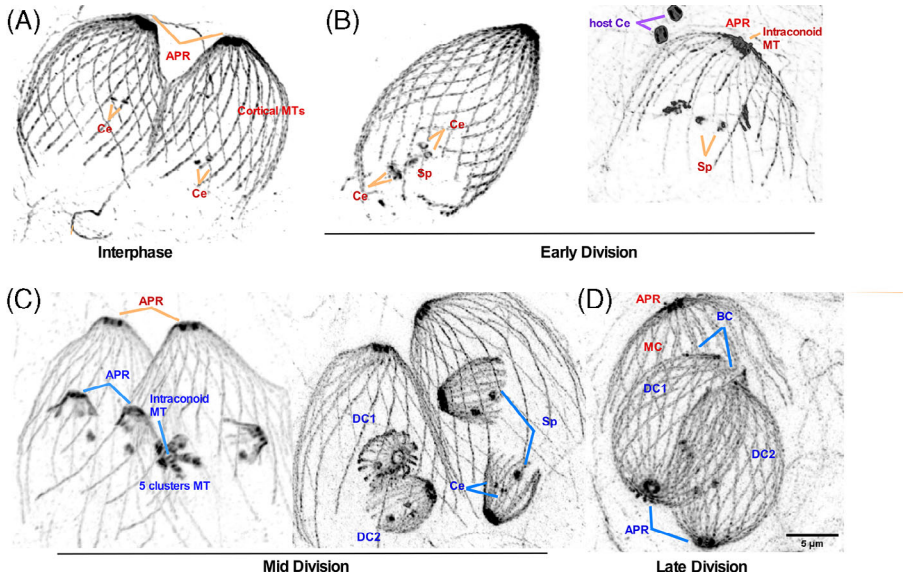

FIGURE 2 **Endodyogeny progression and microtubule-based structures of *Toxoplasma gondii* by ultrastructure expansion microscopy**. U-ExM of tachyzoites was performed following the protocol by Liffner and Absalon.[63] Gel expansion ranges from 4.5- to 5.5-fold. Microtubule-based structures were labelled using anti-acetylated tubulin. Structures highlighted include mother (MC; red), daughter (DC; blue), and host cells (violet). Centrioles (Ce), cortical microtubules, intraconoid microtubules, spindle microtubules (sp), the basal complex (BC), and the apical polar ring (APR). (A) *Toxoplasma gondii* interphase. The two centrioles forming the centrosome can be resolved by U-ExM. (B) Early division of *Toxoplasma gondii*: centrosome duplication and assembly of the spindle pole can be observed. (C) Mid division during endodyogeny, APR from daughter cells appear attached to 5 clusters of MT and the intraconoidal MT can be resolved by U-ExM (D) During *Toxoplasma gondii* tachyzoites division, daughter cells are completely developed, and the BC can be distinguished by U-ExM.

of a daughter cell initiates as an 'imperfect petal' showcasing an array of 22 microtubules arranged in a fivefold symmetrical structure. This had only been observed before using cryo-electron tomography.[74] The enhanced resolution achieved by the additive effects of U-ExM and 3D SIM allowed Padilla and colleagues to resolve that the petal is preceded by 6–7 microtubule stubs, which lay the foundation for the latter assembly of the remaining microtubules, demonstrating for the first time that not all microtubules are initiated at the same time.[72] This study also resolved that the intra-conoid MTs are polymerised slightly after the conoid and cortical microtubule precursors, establishing the temporal sequence of distinct cytoskeletal structures formation. In connection to this, U-ExM has been instrumental in identifying the localisation and function of γ-tubulin and Gamma Tubulin Complex Proteins (GCPs), which are key players in nucleating tubulin structures, including spindle microtubules during mitosis, conoid tubulin fibres, and subpellicular microtubules. These elements are critical for the parasite's cellular architecture and division.[62] U-ExM allowed the characterisation of the early steps in tubulin scaffold formation. In *T. gondii*, γ-tubulin depletion blocked scaffold formation, while depletion of TgGCP4 (γ-tubulin complex protein 4), a previously uncharacterised member of the γ-tubulin complex protein family, disrupted proper SPMT assembly, indicating the importance of the γ-tubulin complex in scaffold formation.[75]

Host cell invasion is aided by cytoskeletal elements which provide the cell with a scaffold upon which membrane components sit, housing proteins and structures critical for motility and secretion. The cortical cytoskeleton is organised by an apical microtubule organising centre known as the apical polar ring (APR). Within the APR rest the apical most end of Rhoptries, known as the rhoptries 'neck'. Rhoptry neck proteins (RONs) are fundamentally involved in the invasion mechanism by forming a moving junction which is held in place by parasite-host membrane connections. By the introduction of U-ExM it was possible to resolve the localisation of RON proteins previously indistinguishable by conventional microscopy techniques.[76] U-ExM confirmed the spatial relationship between two proteins, TgNd6 and TgCRMPs which overlap at the tip of the extruded conoid.[36] Moreover, the localisation and functional significance of two intraconoidal microtubule (ICMT)-associated proteins instrumental for invasion were addressed by U-ExM. Upon knockdown of the ICMT-associated proteins, the ICMT docking at the parasite's apex as well as their architecture is altered, altering in turn rhoptry discharge, affecting parasite invasion. These studies ultimately defined the largely and historically ill-understood relevance of the ICMT.[61]

U-ExM has also provided high-resolution insights into regulatory proteins. Using U-ExM with NHS-ester staining, researchers observed morphological defects like rhoptry disorganisation and plasma membrane dilation at the apical pole in the absence of TgERK7, a kinase essential for the integrity of the apical complex and conoid biogenesis, highlighting its vital role in maintaining proper cellular structure.[77] TgTKL4 is a tyrosine kinase-like (TKL) kinase involved in parasite replication and invasion. TgTKL4 also plays a role in the arrangement of subpellicular microtubules in *T. gondii*. Using U-ExM, defects in subpellicular microtubules were observed in TgTKL4-deficient parasites. These mutants exhibited abnormal morphology, including shorter microtubules, a lack of the typical spiral arrangement, and increased spacing between microtubules.[78] On other hand, an essential kinase of the GSK family, TgGSK, has been shown to critically regulate centrosome-associated functions in cell division and organelle segregation. Its localisation to the centrosome was only highlighted by U-ExM, while undetectable by conventional confocal microscopy.[79] Finally, the characterisation of the plant-like protein phosphatase PPKL, unique to the parasite and absent in its mammalian host, was pursued by U-ExM. PPKL was found throughout the cell in nondividing parasites enriched at the cortical cytoskeleton of forming daughter parasites.[80]

Apicomplexan cell cycle progression is also controlled by cyclin-dependent kinases.[22] *Toxoplasma gondii* Cyclin-dependent kinase-related protein 4 (TgCrk4) and its partner cyclin TgCyc4 were found to play key roles in G2 phase regulation – a phase of the cell cycle largely accepted as absent in this species. TgCrk4 is involved in repressing chromosome re-replication and centrosome reduplication, ensuring proper cell cycle progression acting upstream of the spindle assembly checkpoint.[81] Authors describe by U-ExM that over 90% of TgCrk4-deficient parasites exhibited an altered ratio of centrocone-to-daughter basal complexes (dBC). TgCrk4 depletion also affected apicoplast segregation and fission that occurs concurrently with mitosis. TgiRD1, an interactor TgCrk4–TgCyc4 complex, was also found to control both DNA and centrosome reduplication in tachyzoites.[81] Additionally, U-ExM have been used to follow DNA replication following the lack of a DNA replication factor (TgPCNA1). The mutant showed an arrest of mitotic progression prior to anaphase (separation of the duplicated DNA into daughter cells), a phenotype similar to the lack of a G2 regulator.[82]

Unlike canonical centrosomes which display orthogonally oriented centrioles, *T. gondii* boasts two parallel centrioles, smaller than the canonical ones, located close to the nucleus. Additionally, they display a ninefold symmetry axis composed of microtubule (MT) singlets instead of triplets. Although the detailed spatial and temporal mapping of 24 centriolar proteins in human procentriole assembly was determined by U-ExM,[83] the precise architecture and process of replication is still ill-understood in apicomplexan. In spite of this, the advent of super resolution made it possible to resolve the relative location of different proteins associated to the centrioles or to the mitotic spindle, enabling the proposal of a model for the centrosome encompassing an inner and outer core, whereby each core is associated to a distinct function. The *Toxoplasma* outer core, distal from the nucleus, is bridged from the inner core, proximal to the nucleus, by a middle core.[84] This modular organisation has been proposed to underlie *T. gondii*'s cell division plasticity. By U-ExM, it was possible to determine that TgCep250L1, the only marker known to exclusively localise at the inner core of the *T. gondii* centrosome, rests within the centrocone, an intranuclear compartment devoid of chromatin, whereby mitotic spindle assembly occurs.[85] This observation critically determined that mitosis in *T. gondii* mechanistically resembles that observed in the *Plasmodium* species, whereby an intranuclear spindle is nucleated within a chromatin free region, orchestrated by an extranuclear centriolar plaque.[26]

Using U-ExM it was possible to observe the distribution and localisation of the largely unidentified histone H1 ortholog TgH1-like at different stages of cell division. The protein localises and accumulates within the nucleolus during cell division, while it is detectable at the nucleus periphery at later stages.[86] Together with the identification of a putative H1 homolog, which had been traditionally regarded as nonexistent in *T. gondii*, this mechanistic insight highlights the epigenetic changes accompanying cell division.

Membrane organelles such as mitochondria and apicoplast need to replicate and segregate correctly during parasites division. Although MyoA (a class XIVa myosin motor protein) is known to be located at the parasite's pellicle, U-ExM revealed that MyoA accumulates around the mitochondrion during the late stages of parasite division, a time at which mitochondrial segregation into daughter cells occurs. Parasites lacking MyoA exhibit abnormal mitochondrial morphology and a delay in the delivery of the mitochondrion to daughter parasite buds, indicating that MyoA plays a critical role in organellar inheritance during cell division.[87,88] Using U-ExM for observing endodyogeny in *Neospora caninum*, a close relative to *T. gondi*i, Tomasina and colleagues were able to observe apicoplast segregation into daughter cells, corroborating that in this species the process entails an akin kinetic to that previously observed for *T. gondii* by conventional microscopy.[89]

Although acute infection caused by tachyzoites is the most clinically significant, most cases in immunocompe-

tent individuals are asymptomatic and quickly become latent chronic infections in which the prevalent life stage of the parasite is slow growing.[90] This form is known as the bradyzoite. Chronically infecting bradyzoites, who also divide by endodyogeny, form cysts in muscle and brain tissue. These intracellular cysts, measuring 5–100 μm, have a cyst wall 250–850 nm thick, composed of a granular layer of proteins and carbohydrates within a modified parasitophorous vacuole membrane.[67] Until recently, the cyst wall was resistant to U-ExM, limiting protein localisation studies at this stage, and the detailed studies of its – perhaps distinct – endodyogeny. However, Bondarenko et al. overcame this challenge by introducing a mucinase (StcE) digestion step, enabling full cyst expansion while maintaining fluorescent staining. The protocol of StcEU-ExM was used to localise the GRA2 protein within the cyst wall, offering new insights into cyst biology.[91]

4.2 | Apicomplexan division by endopolygeny

When wild or domestic felids consume bradyzoite-infected tissue or an oocyst from the environment, *Toxoplasma* initiates its sexual differentiation cycle within the cat's intestinal epithelium. Presexual stages precede the formation of gametes. The trajectory towards gamete formation includes the asexual formation of merozoites.[92] Merozoites formation consists of five morpho-types which differentiate to give rise to mature merozoites.[92] These different forms divide by means of endopolygeny, a process involving multiple rounds of DNA replication, followed by karyokinesis, with budding occurring within the cytoplasm (Figure 1). Notably, these life forms have not yet been structurally tackled in detail by U-ExM. Therefore, our understanding of the relative mechanistic and structural differences between the different cell division modes used by *T. gondii* remains to be explored. Nonetheless, light onto the process of endopolygeny under the light of the amped resolution given by U-ExM has emerged from observing *Sarcocystis neurona*, a closely related parasite to *T. gondii*, which also generates life forms via endopolygeny. However, in *S. neurona*, endopolygeny entails several rounds of DNA synthesis without nuclear parcelling, followed by a final round of S- and M-phase linked to internal daughter budding. This process ultimately results in the formation of 64 emerging merozoites.[8] Recently, iterative U-ExM, increasing approximately 13-fold the sample size, enlightened cytoskeleton assembly during division of *S. neurona*. Authors found a close association between microtubules and centrioles, suggesting that an akin structure to the *Toxoplasma*'s centrosome outer core regulates microtubule nucleation.[8]

4.3 | Apicomplexan division by schizogony

Species within the *Plasmodium* genera proliferate through schizogony. Schizogony involves multiple rounds of DNA replication and nuclear division without cytokinesis, resulting in a precursor multinucleated cell. Despite sharing a cytoplasm, the nuclei divide asynchronously. However, a last round of mitosis is followed by the simultaneous formation of numerous daughter cells. U-ExM has advanced the visualisation of mitosis at the level of individual nuclei, revealing structures such as hemi-spindles, mitotic spindles, and interpolar spindles.[63] It has also contributed to understanding the organisation of chromosome-capturing kinetochores in relation to the mitotic spindle, centriolar plaque, centromeres, and apical organelles during schizont development.[93] Proper assembly of the kinetochore/spindle complex is crucial for positioning the nascent apical complex in developing *P. falciparum* merozoites. The apical organelles, such as rhoptries and micronemes, are positioned at the cell's apex and play key roles in host cell invasion, reflecting the coordination between replication and cellular organisation.[93]

Recently, U-ExM has resulted in the most detailed analysis of *Plasmodium* organelle biogenesis to date. In this respect, U-ExM was pivotal in allowing characterisation of the effect of the Rhoptry Neck Protein 11 (RON11) knockdown. Surprisingly, in the absence of RON11, fully developed merozoites display a single rhoptry each. This stand-alone rhoptry in RON11-deficient merozoites was morphologically akin to bundled rhoptries, displaying a bulb and a neck oriented into the apical polar ring. These studies shed light onto rhoptry biogenesis and specie-specific organelle number determination.[94] In addition, several authors have tackled either the dynamics of segregation or de novo generation of several organelles; Atchou et al., 2023 showed mitochondrial dynamics in association with host cell lysosomes by prestaining expansion microscopy (PS-ExM) in *Plasmodium*.[59] Liffner et al. used dye-conjugated reagents and immunostaining to catalog 13 distinct *P. falciparum* structures and organelles across its entire intraerythrocytic development cycle.[64] In the latter, the outer centriolar plaque and associated proteins were found to anchor the nucleus to the parasite's plasma membrane during mitosis. Rhoptries, Golgi, the basal complex, and the inner membrane complex (IMC) form around this anchoring site while nuclear division is still occurring, maintaining association with the centriolar plaque until segmentation begins. The mitochondrion and apicoplast undergo sequential fission events during cytokinesis, also remaining associated with the outer centriolar plaque.

Parasites of the *Cryptosporidium* genera also replicate through schizogony. In this species, the process involves three successive rounds of highly coordinated and synchronous nuclear divisions, resulting in the formation of eight nuclei, followed by segmentation. Despite the critical role of this process in the parasite's life cycle, the biogenesis and organisation of tubulin-based structures, such as the mitotic spindle and microtubules, during *Cryptosporidium* replication remain poorly understood. These structures are key to nuclear division and cell formation, making their study essential for deeper insights into *Cryptosporidium* biology and replication mechanisms. In *Cryptosporidium parvum*, a notable species causing the most serious manifestations of cryptosporidiosis in humans, U-ExM provided valuable insights into its tubulin cytoskeleton organisation and nucleation. Specifically, authors showed the conserved localisation and probable function of γ-tubulin in sporozoites. Interestingly, in γ-tubulin tagged parasites, γ-tubulin protein is undetectable in extracellular sporozoites and in intracellular parasites with 1 nucleus. After nuclear duplication, γ-tubulin was detected forming 2 dots, on each side of the nucleus, near centrin1, a marker for the centrosome in other species. U-ExM revealed that γ-tubulin accumulates on both sides of the spindle microtubules prior to their detection. No accumulation was observed after the budding phase, once the merozoites' cytoskeleton is formed. These findings highlight the conserved mechanism of microtubule nucleation across Apicomplexa.[62]

4.4 | Differentiation of apicomplexans into sexual forms

As previously mentioned, *Toxoplasma* gametes develop within the gut of felids. The gametes can recombine sexually, forming precursors of environmentally resistant unsporulated oocysts that are eventually shed into the environment. Despite their importance for transmission, the challenges and complexities to access to the feline enteric environment in vivo or in vitro translates into limitations to study these forms. Recently, however, transcriptional profiling of merozoites revealed GRA11b, as a marker of this presexual stage.[18,95] And though *Toxoplasma* merozoites remain poorly characterised due to the lack of specific markers to identify, for example, their specific stage of differentiation, major strides towards obtaining sexually dividing forms in vitro have been recently attained, anticipating that these long-inaccessible life stages might be rather sooner than later, accessible and amenable to study.[18,96,97]

In contrast, *Plasmodia* are able to sexually differentiate within accessible compartments of their hosts, such as human/animal blood and the mosquito vector's salivary glands. In this light, Bertiaux et al. characterised the ultrastructure of different stages of the *P. falciparum* gametocyte, ookinete, and schizonts.[60] U-ExM allowed the visualisation, at nanoscale resolution, of the cytoskeletal organisation in developing microgametocytes (5–20 μm), enabling the tracking of the dynamic assembly of axonemes and simultaneous tubulin polyglutamylation in the whole cell. U-ExM also contributed to understanding the diversity of subpellicular microtubule arrays in both merozoites and ookinetes (motile zygote). U-ExM allowed for resolution of the 1 to 2 subpellicular microtubules per merozoite, by fluorescent labelling, which were previously only resolvable by STED[27] and cryo-electron tomography.[98] NHS-ester staining delineated the merozoite shape and highlighted structures with varying protein density, likely corresponding to the APR and the two secretory vesicles known as rhoptries. Differences found between *P. falciparum* and *P. berghei* schizonts support the diversity of apical complex organisation across species within the *Plasmodium*. U-ExM analysis of *P. berghei* ookinetes resulted similarly in stains for α/β tubulin and polyglutamylated tubulin. Notably, U-ExM confirmed the existence of a reduced form of the conoid, thought to be absent in *Plasmodium*, that colocalised with the apical tubulin ring (ATR) in ookinetes.[60] For *P. berghei* ookinetes, U-ExM confirmed that the ATR is not polyglutamylated, which is consistent with previous findings regarding the conoid of *T. gondii*. Notably, some proteins associated with the conoid in *T. gondii* were also conserved in *Plasmodium*, including the SAS-6-like (SAS6L) protein, known to form an apical ring in *Plasmodium* ookinetes. In expanded ookinetes, endogenously tagged SAS6L-green fluorescent protein (GFP) colocalised with the ATR.[60] U-ExM resolved kinetochores along the ookinete meiotic spindle[99] and the dynamic molecular organisation of the bipartite microtubule-organising centre (MTOC), which is regulated by two noncanonical kinases. These findings highlight the role of conserved MTOC components in coordinating mitosis and axoneme assembly, with implication to understand the parasite division and generation of flagella involved in mosquito colonisation and efficient vectorial transmission.[100]

More recently, a novel method called Mosquito Tissue U-Expansion Microscopy (MoTissU-ExM) was developed to expand mosquito tissues, specifically salivary glands and midguts. This technique preserves the ultrastructure of both the mosquito host and any parasites, such as *Plasmodium*, within these tissues. By physically expanding the tissue, MoTissU-ExM enhances the resolution of deep-tissue imaging, allowing for detailed visualisation of oocysts and sporozoites directly in situ, providing critical

insights into host-parasite interactions at a subcellular level.[20]

5 | CHALLENGES, LIMITATIONS, AND FUTURE DIRECTIONS

U-ExM has already revolutionised our understanding of cell biology in many fields. It is a cutting-edge technique that represents a powerful tool for studying the cell division of unicellular parasites based on imaging. U-ExM offers not only unprecedented resolution and detail, but also combines the comparative advantage of being compatible with labelling specific cellular components. U-ExM has bridged the gap between light and electron microscopy, which makes it an invaluable tool for studying the ultrastructure of apicomplexan parasites and their life-cycle transitions, with significant potential for advancing research in parasitology.

Nonetheless, U-ExM is not devoid of limitations. Sample preparation entails two to three full workdays. Postexpansion labelling needs to be assayed and optimised, as not all immunostaining antibodies or fluorescent probes work consistently well. The hydrogel is fragile and sometimes contracts needing to be rehydrated and reassessed for its expansion factor. In addition, due to its properties, gel drifting during image acquisition is not uncommon. Even in light of this, for apicomplexan, it has offered unprecedented insights into their intricate structures and unique and complex mechanisms of cell division, potentially leading to new strategies for combating the diseases they cause.

There are areas of parasite biology that remain unexplored. However, U-ExM continues to evolve rapidly, and combining it with other techniques (such as superresolution microscopy as authors have begun to do) will enable new applications to deepen our understanding of parasite biology. Recent versatile adaptations and improvements to U-ExM have been developed, opening up new possibilities for its application across various systems. For example, Labade and collaborators have recently developed a novel application of expansion microscopy: ExIGS (Expansion Imaging and Genomic Sequencing) which combines two advanced techniques: in situ genomic DNA sequencing and superresolution protein imaging.[101] This powerful integration bridges genomics and imaging, allowing researchers to explore how nuclear and chromatin architecture change in the contexts of disease and aging, opening new avenues for exploration.[102]

Importantly, U-ExM has greatly contributed to equity. Geographical disparities in access to advanced imaging technologies have been ameliorated by U-ExM, as resource-limited settings are finding creative solutions in the optimised use of more accessible but still powerful microscopy, such as confocal or epi-fluorescence. The amped resolution offered by U-ExM can bridge the equipment access gap, enabling the study of *apicomplexans* at super resolution even in resource-limited laboratories worldwide.

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