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Three-dimensional electron microscopy analysis reveals endopolygeny-like nuclear architecture segregation in Plasmodium oocyst development

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**A R T I C L E  I N F O**

**Keywords:** Plasmodium, Oocyst stage development, Nuclear division, Cell division, Endopolygeny, Organelle, FIB-SEM

**A B S T R A C T**

The genus Plasmodium is a unicellular eukaryotic parasite that is the causative agent of malaria, which is transmitted by Anopheline mosquito. There are a total of three developmental stages in the production of haploid parasites in the Plasmodium life cycle: the oocyst stage in mosquitoes and the liver and blood stages in mammalian hosts. The Plasmodium oocyst stage plays an important role in the production of the first generation of haploid parasites. Nuclear division is the most important event that occurs during the proliferation of all eukaryotes. However, obtaining the details of nuclear division at the oocyst stage is challenging owing to difficulties in preparation. In this study, we used focused-ion-beam-milling combined with scanning-electron-microscopy to report the 3D architecture during nuclear segregations in oocyst stage. This advanced technology allowed us to analyse the 3D details of organelle segregation inside the oocyst during sporogony formation. It was revealed that multiple nuclei were involved with several centrosomes in one germ nucleus during sporozoite budding (endopolygeny). Our high-resolution 3D analysis uncovered the endopolygeny-like nuclear architecture of Plasmodium in the definitive host. This nuclear segregation was different from that in the blood stage, and its similarity to other apicomplexan parasite nuclear divisions such as Sarcocystis is discussed.

1. Introduction

Eukaryotic cells have a tightly regulated proliferation with several variations of organelle segregation and are surrounded by a membrane structure. The cell nucleus is one of the most critical organelles in eukaryotes, and its multiplication is controlled by sophisticated mechanisms [1,2]. The nucleus of the eukaryotic cell consists of chromosomes that are surrounded by a double-membrane nuclear envelope. During proliferation, nuclear division occurs and is accompanied either by nuclear envelope disassembly (open mitosis) or the maintenance of its intact structure before chromosome segregation (closed mitosis) [3]. Open mitosis has been observed in higher multicellular eukaryotes, and chromosome segregation is accompanied by nuclear envelope disruption via several similar regulation mechanisms [1,2,4]. On the contrary, closed mitosis has been observed in unicellular organisms such as protozoan parasites, and it is considered the most ancient mechanism of eukaryotic cell division [5]. However, because the mechanisms of nuclear division during segregation in closed mitosis are highly divergent from each other, knowledge of the molecular machinery is limited, particularly on how nuclear envelope segregation is controlled during...
nuclear divisions in protozoan parasites [6–12].

*Plasmodium* is a genus of unicellular eukaryotic protozoan parasites that are the etiological agents of malaria, which is transmitted to mammalian hosts by the Anopheline mosquito. The life cycle of *Plasmodium* development comprises two proliferation stages in humans (liver and blood stages) and one proliferation stage in *Anopheles* (oocyst stage). Anopheline mosquitoes spread the infectious *Plasmodium sporozoites* (SPZs) by puncturing the human skin to feed on blood. Upon entering the bloodstream, the SPZs soon accumulate in the liver, where they invade hepatocytes to start liver stage development [13]. Merozoite production occurs at the end of liver stage development, and the released merozoites invade red blood cells to cause symptomatic blood stage development by continually increasing in numbers. The proliferation during blood stage development involves closed mitosis, with the nuclear envelope maintaining its intact structure [14–16]. Nuclear division by closed mitosis occurs in all three proliferation stages of *Plasmodium* species before dividing into daughter parasites. However, information regarding nuclear envelope division, particularly multinuclear segregation within one parasite, remains to be unveiled.

The multinuclear segregation of blood stage parasites is called schizogony formation, which can be analysed in detail by using several types of light and electron microscopes. Blood stage development in closed mitosis has been observed using indirect immunofluorescence analysis and reveals nuclear division into two nuclei, similar to the process of closed mitosis in yeasts [14–16]. Several studies using transmission electron microscopy (TEM) and a limited number of studies using an advanced technology-focused ion beam (FIB) milling combined with scanning electron microscopy (SEM) have clearly shown the structure of the divided parasites [17–20]. It is difficult to show the processes in the middle of nuclear division (e.g., when the single nucleus divides into two nuclei) because the *Plasmodium* parasite is at its smallest size in the blood stage. Oocyst stage development has been observed using TEM or SEM, some of nuclei appeared in micrographs as irregular and elongate. Unfortunately, it was concluded that there were occasional invaginations of nuclear membrane due to the classical low-resolution micrographs with only 2D analysis [21,22].

The genus *Plasmodium* belongs to the phylum Apicomplexa, which includes genera *Toxoplasma*, *Cryptosporidium*, *Eimeria*, *Isospora* and *Sarcocystis*. The nuclear division of these apicomplexan parasites has been found to be extraordinarily divergent depending on each parasite, and a possible change in the mechanism of nuclear division during each developmental stage has been reported for some of them [23–26]. It is known that the proliferation of *Sarcocystis neurona* occurs via endopolygenesis, wherein multiple nuclei are divided from one germ nucleus [27]. On the contrary, endodyogeny has been observed in the proliferation of *Toxoplasma gondii*, in which two daughter cells are produced inside the mother cell with closed mitosis [28–30]. It was reported that the sexual developmental stage of *Toxoplasma* in the definitive host undergoes endopolygenesis [31]. Thus, variations in the mechanisms of nuclear division according to the parasite developmental stages are suggested in Apicomplexa.

In this study, we report detailed morphological differences in the oocyst stage development between *Plasmodium* species in the definitive host (Anopheline mosquito). Moreover, we present a 3D structural analysis of the nuclear divisions involved in endopolygeny-like segregation in oocyst stage development, in which multiple nuclei were connected with several centrosomes in one nucleus during the immature oocyst stage. Electron microscopy analysis revealed that *Plasmodium cynomolgi* (PCy) oocyst stage development had a greater advantage in terms of cellular biology in the observation of organelle segregations. And also PCy would be attractive target for future study due to the dormant formation (called hypano zoites) in the liver stage development. The differences between the nuclear division mechanisms at the oocyst stage and schizogony at the blood stage are discussed.

2. Material and methods

2.1. Animal experiments and ethical committees

Two female Japanese macaques (5 years old; weighing 4.0–4.5 kg) were used for the experiment. The monkeys (*Macaca fuscata*) were provided by the National Bio Resource Project “Japanese macaques” at Kyoto University Primate Research Institute (Aichi, Japan). They were second-generation offspring bred in captivity, and they were bred and grown at animal facilities in a malaria-free environment in Japan. The infectious experiments for the monkeys were performed under biosafety level 2 conditions at the Tsukuba Primate Research Center (TPRC), National Institute of Biomedical Innovation, Health and Nutrition, Ibaraki, Japan. The investigators adhered to the Guidelines for the Use of Experimental Animals authorised by the Japanese Association for Laboratory Animal Science. The protocol was approved by the Committee on the Ethics of Animal Experiments of the National Institute of Infectious Diseases Tokyo, Japan (for rodent experiments, permit number: 117108-II-2); Dokkyo Medical University, Tochigi, Japan (for primate experiments, permit number: 1007); and the TPRC (for primate experiments, permit number: DS29–14). The monkeys were used under the supervision of the veterinarians in charge of the animal facility.

2.2. Parasites and reporter parasites used

The *P. cynomolgi* B strain was obtained from ATCC (ATCC No. 30129) and maintained in Japanese macaques housed in the animal facility of Dokkyo Medical University, Japan. We used the rodent malaria parasites *Pb* and *Py*, which are the most common parasites utilised globally in experimental malaria models, transgenic *PbEF-GFP::Luc* (676m1cl1) and *PyGFP::Luc* (171). Information on the rodent parasite line can be found in the Rodent Malaria Genetically Modified Database (https://www.pbergehi.eu/index.php).

2.3. Mosquito experiments for oocyst and SPZ sample preparation

The laboratory-bred *Anopheles stephensi* mosquitoes were used for oocyst and sporozoite generation in this study. The colony of *An. stephensi* had been maintained in the Department of Parasitology, National Institute of Infectious Diseases (NIID), Japan (Tokyo, Japan). To develop oocysts and SPZ in mosquitoes, *P. cynomolgi* B-infected blood was obtained from the donor monkey and fed into approximately 200 mosquitoes using the standard artificial membrane feeding method [32]. The fed mosquitoes were kept in climate chambers at 24 °C with 80% humidity and fed regularly with 5% glucose-soaked cotton. The *Pcy*-infected mosquitoes were maintained in an incubator under BSL-3 condition at TPRC. Infection of mosquitoes with rodent parasites (*Pb* and *Py*) was done using canonical standard method under BSL2 condition at NIID [33]. Oocysts were obtained from dissected midgut at day 14–21 after blood-feeding, and SPZs were obtained from SGs on days 20–28. After collection, the midguts and SGs were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C. The total number of mosquitoes required was dependent on the experiment and the number of SG SPZs (and oocysts) per dissected mosquito.

2.4. Sample preparation for SEM

For SEM, the organs were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4 °C, followed by postfixation with 1% OsO4 in the same buffer. Thereafter, the fixed specimens were dehydrated in a graded series of ethanol. Then the ethanol was replaced with liquid CO2 in the chamber of a critical point dryer (CPD 030; BAL-Tec), and the specimens were dried by critical point drying. After mounting on aluminium stubs with
carbon paste, the dried specimens were coated with osmium by using a plasma coating device (Neoc-5T; Meivuafosiu) and then observed with a scanning electron microscope (SU6600; Hitachi). The diameter of oocysts were measured using a measurement software (Quartz PCI 9; Quartz Imaging Corp).

2.5. TEM and serial block-face imaging by FIB-SEM

For TEM, the organs were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde (TAAB Laboratories Equipment Ltd.) in 0.1 M phosphate buffer (pH 7.4) overnight at 4 °C, followed by postfixation with 2% OsO4 in the same buffer. Thereafter, the fixed specimens were dehydrated with a graded series of ethanol and embedded in Epoxy 812 (Oken Shoji). Ultrathin sections were cut and stained with uranyl acetate and lead citrate and were then examined using an HT7700 transmission electron microscope (Hitachi).

For the serial block-face SEM of oocysts, images were taken using FIB-SEM on a Helios NanoLab 660 (FEI). The Epon blocks of midguts were milled using a FIB, and the serial block-face imaging of the oocysts was performed every 40 nm by using a backscattered electron detector (MD detector) at an acceleration voltage of 2.0 kV and a current of 0.4 nA.

For the classical 3D analysis using an array tomography of the SPZs, the ultrathin serial sections of the SGs (70 nm thick) were cut, mounted on a glass coverslip and stained as described above. Thereafter, the sections were coated with osmium at a thickness of 2 nm by using an OPC80T osmium plasma coater (Filgen) and then observed using FIB-SEM on a Helios NanoLab 660. The 3D reconstruction was performed using Amira software (FEI).

3. Results

3.1. Morphological differences between rodent malaria parasites in oocyst stage development

Studies are lacking on the comparative analysis of the species-specific morphological differences in Plasmodium oocysts using the same Anopheles species (Anopheles stephensi). To observe the morphological differences in the Plasmodium oocyst stage, we employed two rodent malaria parasites, P. berghei (Pb) and P. yoelii (Py), for SEM analysis (Fig. 1A and B). The infected mosquito midguts and salivary glands (SGs) were collected under microscopy by hand dissection on several days from 14 to 21 days post-infection (dpi). To define the criteria for oocyst maturation, we counted the number of oocysts in the midgut and SPZs in the SG. Several hundred oocysts were observed under light microscopy with green fluorescent protein (GFP) positive in both Pb and Py (Fig. 1Aa–band Ba–b), and fully mature oocysts were observed at 21 and 14 dpi for Pb and Py, respectively. These time points have previously been confirmed as the start of SPZ accumulation in the SG [32]. Our SEM analysis clearly demonstrated the morphological differences between Pb and Py oocysts in both the diameter and thickness of the layer from the basal surface of the midgut. We employed the same Anopheles stephensi (An. stephensi) to set up the infection for Pb and Py, and most of the oocysts of both parasites were covered by the muscle tissue of the midgut (Fig. 1Ad–f and Bd–f). Our observations revealed that the major difference between Pb and Py oocysts occurred in the distal portion of the protrusion from the midgut tissue (Table 1), wherein the Py oocysts bulged out more from the midgut than Pb oocysts. The surface of the Pb oocysts was observed as a smooth membrane structure (Fig. 1Ad and Ae), but some Py oocysts demonstrated a membrane structure with a thorny appearance (Fig. 1Bd and Be). The difference in the surface structure appeared to be species-specific and was not associated with maturation because the number of Py oocysts with a thorny appearance did not increase. The mature parasites SPZs were observed by the start of budding and the initiation of the release of SPZs from the oocysts (Fig. 1Ag and Bg).

3.2. Rodent malaria parasites in the SG, the quality of organelle resolution was insufficient by classical 3D structure analysis

After the mature SPZs are released from oocyst, they accumulate in the SG, which is the final platform for malaria parasites in the mosquito stage before infection into a mammalian host. We employed transgenic GFP-expressing Pb to visualise SPZ accumulation in the SG lobes, and a strong GFP signal was detected only in the lateral lobes in Pb-infected An. stephensi at 21 dpi (Fig. 2Aa and Ab). We performed SEM analysis of the SGs to observe in detail the morphological differences between each lobe, and intumescence and swelling of the lateral lobes were clearly observed (Fig. 2Ba and Bb). Moreover, some of the lateral lobes contained SPZ (Fig. 2Bc) and numerous SPZs on their surfaces (Fig. 2Bd). To elucidate the details of SPZ accumulation in the SG, we performed classical array tomography of the Pb-infected SG lobes and found the typical morphology of SPZ accumulation in the lateral lobes. Moreover, the majority of SPZ accumulation was observed in the area of the epithelial cells, and very few SPZs were found in the secretory cavity and duct area (Supplementary Figs. 1a and 1b).

To visualise the 3D structure of SPZ accumulation with the architecture of individual organelles, we performed classical array tomography (Fig. 2Cb). We successfully utilised this analysis to determine the 3D structure of the SPZ parasite (Fig. 2Ca) with 70 nm slice staining; however, our classical 3D structure for SPZ was clearly shown artefacts especially nuclear and apical end of SPZ with serious gap (Fig. 2Cb. White arrow), the quality of organelle resolution was insufficient for performing further analyses, particularly the analysis of organelle segregation and localisation.

3.3. The protruding oocyst with a smooth membrane appearance was connected to the mosquito basal membrane in P. cynomolgi

We succeeded in optimising the conditions for a high rate of infection for several Plasmodium species by using the same An. stephensi. We collected a large number of P. cynomolgi (Pcy)-infected An. stephensi midguts at 21 dpi (Fig. 3a) and fixed them for SEM analysis to observe the mature fully oocysts (Fig. 3b–j). The diameter and area of the Pcy oocysts were 17.4 ± 1.3 μm and 961 ± 143 μm², respectively (Table 1). These results clearly indicated that the Pcy oocysts were larger than the rodent parasites (Fig. 3b, e and f). Furthermore, basal-membrane-connected oocysts were frequently observed in Pcy (Fig. 3c, d and g. White arrowheads). No major difference in the production number of oocysts/mosquitos was observed between all three Plasmodium species (Pb, Py and Pcy), but Pcy species-specific differences were noted. Some of the fully matured Pcy oocysts with small cracked walls showed the ongoing budding out of SPZs inside the oocysts (Fig. 3h and i). We found that hundreds of SPZs were still connected to the midgut by a basal residual core body (Rb) (Fig. 3i and j), and this is known as sporogony formation. The size of the Rb shrunk when SPZs matured during budding (Fig. 3i and j). The sporogony form of parasites was observed in one oocyst, and these forms were separated and packed against each other. Our SEM analysis of Pcy oocysts revealed that this was the best timing to observe the production of SPZs for the future investigation of organelle segregation.

3.4. The 3D analysis of immature oocyst reconstruction of organelle architectures revealed endopolygy-like nuclear segregation in P. cynomolgi

Multinuclear segregation has been well studied in blood stage parasites, wherein the parent nucleus divides into two daughter nuclei without the segregation of the plasma membrane of the parasite. This is known as schizogeny development [16,25]. To observe the details of nuclear segregation at the oocyst stage so that the differences from schizont segregation could be determined, we analysed Pcy-infected midguts, which is the largest and longest period to generate SPZ productions, by using FIB-SEM. This is one of the best options for the monitoring of nuclear division. Pcy-infected midgut was dissected at 14
Fig. 1. Morphological differences of rodent malaria parasites in the oocyst stage of development. A. Fully mature Pb-oocyst development in the mosquito midgut at 21 dpi (Pb; 676m1c1l). B. Fully mature Py-oocyst development in the mosquito midgut at 14 dpi (Py; 1971). Visualisation of the infected mosquito midgut. A, B. (a) Bright-field microscopy, (b) GFP fluorescence signal (488 nm). (c–g) Observation of the infected mosquito midgut using SEM. (g) Newly hatched SPZs from oocysts were observed. White arrowheads, oocyst; Yellow line, midgut. The scale is shown in each image. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
dpi, which was expected to be the middle of the proliferation period and is when the most drastic changes of organellar segregation occur. The images of semithin section slides with toluidine blue staining clearly showed the oocyst morphology (Fig. 4Aa. Black square), and this was confirmed by TEM analysis as sporogony formation (Fig. 4Ab and Ac). It was easy to recognise the apical ends of the SPZs because of the high electron-dense of plasma membrane and rhoptries (high-electron-dense organelle). These observations clearly indicated that the state of these oocysts was still immature because the SPZ plasma membrane (Fig. 4Ac. Black line) was still bonded, and many rhoptries were located in the same budding SPZ (Fig. 4Ab and 4Ac). To reveal the details of organelle segregation in SPZs production, we performed FIB-SEM analysis by using the immature oocyst. Our analysis showed that most of the immature SPZ contained several rhoptries (Fig. 4Ac, 8a and Cb. Green arrowheads) that are located near the apical end of the plasma membrane. The apical ends of the SPZs were not yet well protruded because the budding parasites are still incomplete, and we could also observe Rbs in the centre of the sporogy formation of these immature parasites (Fig. 4Ac). To clarify the nuclear segregation of immature oocyst, we conducted a 3D analysis of these parasites. We obtained approximately 200–500 serial block-face of images/oocyst, and sporogony formation parasites that contained rhoptries and the whole nucleus within a series of slices were selected for 3D analysis. The nuclei of these parasites were also found to be centrosome positive, as evidenced by a high electron-dense with a typical centrosome shape on the edge of the nuclear envelope. A series of slides revealed that these centrosomes were located on the same nucleus (Fig. 4B slices 130 and 132. Orange arrowheads). The segmentation of each organelle for 3D analysis was performed by colour staining. Fig. 4Bb and Cb shows the 3D reconstructed images and videos (Movies. 1 and 2). These videos clearly indicated that the nuclear envelope of immature oocyst was bonded with multiple centrosome-positive nuclei (Movie 1), and this process is similar to endopolygony. Moreover, in the simple and clear examples of multiple centrosome-positive nuclear images that we obtained (Fig. 4Ca, Cb and Movie 2), nuclear division showed an endopolygony-like structure that was unmistakably different from that of schizonts. This was the first demonstration of Plasmodium endopolygony-like nuclear architecture in the definitive host.

3.5. The mature SPZ were observed inside oocysts with a divided nucleus accompanied by sophisticated organelle distribution

Our 3D-electron microscopy analysis of immature oocyst nuclear architecture revealed that endopolygony-like segregation occurred in Plasmodium cynomolgi in the definitive host for this parasite. To follow up on this unique nuclear segregation, we performed the same analysis at a later time point of oocyst stage development. At 21 dpi, Pcy-infected Anopheles midguts were dissected to observe the morphological differences of parasite development regarding organelle segregation and distribution, particularly the nuclear architecture. Our TEM analysis (Fig. 5A) revealed that the SPZ plasma membrane structures were well partitioned into the divided SPZs, with several intact mature organelles, such as rhoptries. Furthermore, large size of Rb was difficult to find at this time point. These observations indicated that the fully mature and final stage of oocyst development to produce the SPZs occurred at 21 dpi. The FIB-SEM analyses of several Pcy-infected mosquito midguts were performed using serial sections of 150 images per 40 nm, and Fig. 5B presents the typical images. Enlarged and well-parcellated mature rhoptries were observed inside SPZs as highly electron-dense organelles (Fig. 5Ba. Green arrowhead) accompanied by distributed nuclei. Alternative or immature organelles are distinguishable from mature rhoptry (AOR), which are clearly different from mature rhoptries because of a different electron-dense and are surrounded by a double membrane, were observed between the mature rhoptries and the nucleus (Fig. 5Bb. Blue arrowhead). AOR was not observed in the immature oocyst at 14 dpi. The elongated parasite nucleus was well partitioned and divided into each nucleus one by one for SPZ generation. The 3D reconstructed structure images (Fig. 5Ca and Cb) clearly showed the organelle distributions of the divided SPZs and demonstrated the orchestrated ordered assembly for each organelle (rhoptries, AORs and nucleus) (Fig. 5C, Movies 3 and 4). The centrosome structures were not observed in well-partitioned parasite nuclei at this time point. Centrosomes are essential only for chromosome distribution and segregation; therefore, these results indicated that the parasite had already completed these nuclear segregation processes.

The schematic diagrams of nuclear division in Plasmodium species and Sarcocystis neurona were shown in Fig. 6. It has been reported in Plasmodium that schizogyony formation was observed as multinuclear segregation in blood stage parasites. In the schizont stage, the mother nucleus divides into two daughter nuclei, and this process of multiple nuclear fission was continually repeated [14–16]. The current study is the first report of endopolygony-like proliferation in Plasmodium oocyst stage development with the mother nucleus divided into more than three nuclei at the same time, which is similar to the Sarcocystis nuclear division [27].

4. Discussion

In this study, we revealed the occurrence of endopolygony-like nuclear architecture segregation in Plasmodium oocyst development. This was similar to the nuclear segregation reported in another apicomplex parasite, Sarcocystis neurona. Our 3D FIB-SEM analysis clearly showed that the immature oocyst mother nucleus was divided into several daughter nuclei that were joined by a network formation. Analysis using 3D FIB-SEM is advantageous because it can resolve organelle structure, particularly the high resolution of the membrane architecture of each organelle, in far greater detail than other techniques. It was difficult to obtain a high resolution of the 3D structure of organelles by using the classical array tomography method. Although we had previously employed this method of classical 3D analysis for the reconstruction of SG SPZs, it was challenging to obtain an accurate 3D reconstruction of organelle structure because of the thickness limitation of the serial sectioning slices (Fig. 2Cb). There are a limited number of articles that detail the 3D architecture of organelles by FIB-SEM for the Plasmodium blood stage because of the small size of the parasite [17–20]. These FIB-SEM studies have mainly focused on the architecture of parasite-specific organelles in the blood stage, and none of them reported on the other developmental stages.

We need to ask why the endopolygony-like formation in Plasmodium was not previously discovered. Some of classical EM articles had been mentioned occasional invaginations of nuclear membrane with irregular and elongate shape in oocyst stage development. However, unfortunately it was difficult to conclude as in endopolygony-like structure due to only performed the classical 2D analysis [21,22]. Although advanced 3D FIB-SEM analysis has made it possible to obtain the high-resolution images of nuclear architecture, this was previously difficult to obtain using classical array tomography 3D analysis. Another possibility is that the confocal microscopy images using Hoechst or 4′,6-diamidino-2-phenylindole DNA staining in the oocyst stage could have been misleading. These DNA staining methods bind with a high affinity to the minor groove of AT-rich DNA sequences. It has been reported in

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<th>Average ± SD</th>
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<tr>
<td>Average ± SD</td>
<td>Area (μm²)</td>
<td>Diameter (μm)</td>
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<tr>
<td>P. berghei</td>
<td>207 ± 36</td>
<td>8.1 ± 0.7</td>
</tr>
<tr>
<td>P. yoelii</td>
<td>418 ± 103</td>
<td>11.4 ± 1.4</td>
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<tr>
<td>P. cynomolgi</td>
<td>961 ± 143</td>
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several articles that Hoechst signals with dots were shown in nuclei in the late oocyst stage of development. However, these signals did not show all the nuclear areas, particularly during nuclear division, because the signals became weak or disappeared when the DNA became relaxed.

To visualise the nuclear segregation, we generated a reporter parasite line with SAS6 (spindle assembly abnormal protein 6 homolog) c-terminal tagging by mCherry in Pb. SAS6 is one of the best markers for centrosomes and is recruited to centrioles at the onset of the centrosome duplication cycle [28]. Although strong SAS6–mCherry signals were detected in the oocyst stage, most oocysts with a strong SAS6–mCherry signal showed an undetectable level of Hoechst signal. On the other hand, oocysts without a strong Hoechst signal showed an undetectable level of SAS6–mCherry signal (Supplementary Fig. 2C). It is suggested that SAS6–mCherry-positive oocysts were undergoing nuclear division and that SAS6–mCherry-negative oocysts had completed nuclear division. These unexpected results led us to the realisation that the shape of the oocyst nucleus might not be reflected by the Hoechst signal (Supplementary Fig. 2C), and future experiments are necessary to
Fig. 3. Observation of the oocyst in Pcy. Fully mature Pcy-oocyst development in mosquito midgut at 21 dpi. Visualisation of the infected mosquito midgut. (a) Bright-field microscopy. (b–j) SEM. (f–j) High-magnification images of the oocyst. (g) Each hatched oocyst was connected by the muscle tissue of the mosquito. (h–j) Sporogony formation was observed inside mature oocysts. White arrowheads, basal-membrane-connected oocyst; Rb, residual core body. The scale is shown in each image.
clarify these possibilities. Our 3D FIB-SEM analysis revealed not only the endopolygeny-like nucleus in the oocyst stage of development but also the appearance of AORs. Owing to the difference in electron density, differentiating AORs from rhoptries was easily performed. Several additional experiments are required to determine the nature of specific organelles, and it is possible that AORs could be immature rhoptry, pro-micronemes, immature dense granules or other unknown organelles [34]. The 3D FIB-SEM analysis of the oocysts of rodent malaria parasites on several different postinfection days could provide more information.

Endopolygeny nuclear segregation that involves multiple rounds of DNA replication without nuclear division and internal budding within the cytoplasm has been observed in certain species of *Sarcocystis* [27] (Fig. 6). Nuclear division via both endodyogeny and endopolygeny has been reported in *Toxoplasma* (Supplementary Fig. 3). Endodyogeny is the formation of two daughters within the mother cell cytoplasm before the completion of nuclear division. *Toxoplasma* endopolygeny nuclear division was observed in the definitive host, which involved a proliferative phase with repeated nuclear division [28–30]. A variety of nuclear division mechanisms are often found in these apicomplexan parasites according to the developmental stages, particularly during proliferation in the definitive or intermediate hosts. Considering that both endodyogeny and endopolygeny nuclear division mechanisms have been reported in intermediate and definitive *Toxoplasma* hosts, this could be related to haploid parasite generation. These observations suggested that the difference in proliferation systems occurred during the stage transition from diploid to haploid in the definitive host [31]. However, the importance of endopolygeny formation in *Toxoplasma* remains under discussion.

Our analysis revealed the endopolygeny-like nuclear architecture in the mosquito oocyst stage of *Plasmodium cynomolgi*, and this nuclear architecture was clearly different from the schizont stage that occurs in host blood. Therefore, the mechanisms of nuclear division in *Plasmodium* species might change according to the developmental stage. To reveal the nuclear division system in all stages of the *Plasmodium* life cycle, 3D FIB-SEM analysis needs to be performed on the liver stage of development. One of the most challenging questions is how to control nuclear segregation at the liver stage, where the formation of dormant hypnozoites occurs. Therefore, the mechanism of nuclear regulation at the liver stage in *Plasmodium* species would be an attractive subject, particularly because it has been reported that the nucleus in hypnozoites remains as a single form during dormancy. However, because of
difficulties in setting up the hypnozoite stage of development, the number of hypnozoites would be limited. The targeting of hypnozoites for malaria elimination could shed light on a new strategy to discover novel antihypnozoite drugs that act via the mechanism of nuclear division in the future.

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Author contributions

TAR and TAN designed the experiments. TAR, SKW, YU, YSN and TAN performed the experiments and analysed the data. TAR, SKK, HK...
and TAN performed the electron microscopy and analyses. SKW, TS, KN, YY, TN, HH, BFF and SMK provided valuable materials and conceptual input as well as edited the manuscript. TAR and TAN prepared the manuscript. All authors read and improved the data analysis and approved the final version of the manuscript.

Declaration of Competing Interest

The authors have declared that no competing interests exist.

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