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Full maturation of in vitro *Plasmodium falciparum* oocysts using the AlgiMatrix 3D culture system

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Abstract

Background *Plasmodium falciparum* oocysts undergo growth and maturation in a unique setting within the mosquito midgut, firmly situated between the epithelium and the basal lamina. This location exposes them to specific nutrient exchange and metabolic processes while in direct contact with the mosquito haemolymph. The limited availability of in vitro culture systems for growth of the various *P. falciparum* mosquito stages hampers study of their biology and impedes progress in combatting malaria.

Methods An artificial in vitro environment was established to mimic this distinctive setting, transitioning from a 2D culture system to a 3D model capable of generating fully mature oocysts that give rise to in vitro sporozoites.

Results A two-dimensional (2D) chamber slide was employed along with an extracellular matrix composed of type IV collagen, entactin, and gamma laminin. This matrix facilitated development of the optimal medium composition for cultivating mature *P. falciparum* oocysts in vitro. However, the limitations of this 2D culture system in replicating the in vivo oocyst environment prompted a refinement of the approach by optimizing a three-dimensional (3D) alginate matrix culture system. This new system offered improved attachment, structural support, and nutrient exchange for the developing oocysts, leading to their maturation and the generation of sporozoites.

Conclusions This technique enables the in vitro growth of *P. falciparum* oocysts and sporozoites.

Keywords *Plasmodium*, Ookinetes, Oocysts, 3D matrix, Alginate sponge, Sporozoites

Background

Malaria is a vector-borne disease caused by *Plasmodium* parasites and transmitted by mosquitoes. The burden of malaria is enormous and remains a significant global health problem [1]. Development of reliable vaccines and drugs against human malaria depends on the ability to study both the mosquito and human host cycles of the parasites.

The current WHO-recommended malaria vaccines, RTS,S/AS01 and the related R21, provide partial protection in young children [1, 2], but fail to confer sufficient long-lasting protection against the disease. Another vaccination approach is administration of attenuated

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(irradiated or genetically modified) *Plasmodium falciparum* whole organisms (WO), specifically sporozoites (SPZ), the infectious form of the malaria life cycle. SPZ injected as a vaccine provide a wider repertoire of antigens than subunit vaccines. However, availability of SPZ is limited by the expensive and tedious process of harvesting them from mosquitoes. Yields of genetically-attenuated parasites in particular are modest [3–5]. Growth of SPZ in culture would allow streamlining and scaling of WO production, vastly expanding yield and reducing cost. Currently, Sanaria™, a US-based biotechnology company, is one of the few that can produce in vitro PfSPZ in culture, but their process relies on extraction of aseptic and purified SPZ from dissected sterile mosquitoes. Thus the quantities of SPZ needed for mass-vaccination campaigns are not yet available.

Culturing mosquito stages of *P. falciparum* in vitro could also stimulate research. Culture of both the human and mosquito stages has been complicated by the parasite's stage-specific physiological and nutritional requirements. Most of the research successes that have been achieved in in vitro culture of *P. falciparum* strains yield the exoerythrocytic stage (within liver cells) and the erythrocytic stage (within red blood cells), but these studies have been limited to the sporogonic events that occur within the mosquito vector. *Plasmodium falciparum* parasites in vivo undergo fertilization and sporogony to produce infectious SPZ, thus allowing further transmission [6]. As the female *Anopheles* mosquito takes up a blood meal from an infected individual, consumed microgametocytes (male gametocytes) and macrogametocytes (female gametocytes) sense changes in the mosquito midgut microenvironment and trigger sexual reproduction. The microgamete penetrates the macrogamete, generating a zygote [7], which transforms into a motile and invasive ookinete within 24 to 36 h [8]. During the sporogonic cycle, these ookinetes burrow into the midgut and attach near the basal lamina and epithelial cells while in contact with the mosquito hemolymph to mature into oocysts. The oocysts develop for approximately 10–14 days, eventually rupturing and releasing thousands of SPZ into the midgut haemolymph.

Ookinete development is a major bottleneck in the parasite life cycle and remains a primary target stage for development of transmission-blocking vaccines [9, 10]. Most advances in understanding ookinete biology have been based on studies of *Plasmodium* strains that do not infect humans, such as the avian strain *Plasmodium galinaceum* [11] and the rodent strains *Plasmodium berghei* [12–15] and *Plasmodium yoelii* [16]. The first reports of *P. berghei* ookinete development in vitro were published in the 1960s [12–14]. However, very few reports have described in vitro production of the *P. falciparum* oocyst

and SPZ stages [17]. Development of the *P. falciparum* ookinetes has been studied with limited success, suggesting possible differences in the metabolic organization of the mosquito stages between parasite species [18, 19].

Producing the various *P. falciparum* mosquito stages in vitro without contamination by mosquito debris has proven difficult, but improvements have led to successful growth of the upstream stages, including gametocytes and zygotes [18, 19]. Culture of the *P. falciparum* NF54HT-GFP-luc gametocyte has been demonstrated by many researchers [20, 21]. Previous work [8] described the induction and purification of zygotes from gametocyte culture, leading to the formation of ookinetes, the precursor to the oocyst. However, replicating the microenvironments and interactions of the parasite with the epithelial cells, basal lamina, and haemocoel of the mosquito downstream of the zygote stage is much more challenging. Development of mature oocysts and SPZ in a manner that recapitulates growth in the mosquito requires a myriad of nutrients and environmental factors that have rendered in vitro culture of these stages difficult.

This manuscript presents results of testing a range of culture conditions for the development and maturation of oocysts in a two-dimensional model using a cellular matrix. We subsequently optimized a three-dimensional model using AlgiMatrix (alginate sponge) for the maturation of *P. falciparum* oocysts, leading to in vitro SPZ production.

Methods

Blood stage culture

Plasmodium falciparum NF54 expressing both GFP and luciferase (NF54HT-GFP-luc) [21] was maintained at 5% haematocrit in RPMI 1640 containing 2 mM L-glutamine (R6504, Sigma, MO) supplemented with 10% v/v A + human serum (pooled, heat-inactivated, Valley Biomedical, VA), 25 mM HEPES (H4034, Sigma, MO), and 50 μ M hypoxanthine (H9636, Sigma, MO) at 37 °C under 5% CO₂, 2% O₂, and 93% N₂. The culture medium was replaced daily, and the media were divided when parasitaemia reached 3%.

Gametocyte and zygote cultures to ookinete induction

Gametocyte cultures were initiated from asexual parasites at 2% parasitaemia and 5% haematocrit. Cultures were maintained under the same conditions as described above for 14 days with daily medium changes without splitting. Exflagellation was determined from day 12 to day 14 by spinning 200 μ l of culture at 10,000 \times g for 3 min. After removal of the supernatant, the pellet was resuspended in 50 μ l of ookinete medium and counted on a haemocytometer after allowing the mixture to rest

for 15 min. A gametocyte culture was considered to have matured when more than one exflagellation was observed in 20 random microscopic fields of view [19, 22]. Mature gametocytes were centrifuged at 800×g for 5 min (9 accelerations, 3 brakes) at room temperature (RT). To induce zygote formation, the pellet was resuspended in ookinete medium and incubated at 27 ± 2 °C in an incubator for 24 h. The ookinete medium was RPMI 1640, Schneider's (S0146, Sigma–Aldrich, MO), and Waymouth's (11–220-035, Thermo Fisher Scientific, US) media at a 1:1:1 ratio along with 20% v/v fetal bovine serum (FBS), 4% v/v human red blood cell (RBC) lysate, 0.04% w/v NaHCO₃ (BDH9280, VWR, US) and 0.25% w/v trehalose (AC309870250 Thermo Fisher Scientific, US), adjusted to pH 7.4 at RT [22], sterile-filtered and stored at 4°C.

In vitro oocyst culture in a 2D chamber

Ookinetes were purified by LS MACS columns (Miltenyi Biotec, Bergisch Gladbach, Germany) with an attached 24-gauge flow restrictor (Strategic Applications, IL) mounted on the QuadroMACS Separation system (Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described [22]. After purification, ookinetes were counted under a microscope with a hemocytometer, and 20,000 ookinetes were seeded in oocyst medium within a 2D culture system composed of an 8-well chamber slide (154,941, Electron Microscopy Sciences, PA) coated with 50 µg/ml collagen IV (354,233, Corning, NY) and 25 µg/ml laminin/entactin (ECL) (354,259, Corning, NY). The slides were incubated at 27 ± 2 °C in an incubator for up to 25 days.

Four oocyst media (A, B, C, and D) were tested. Oocyst medium was changed every 3–5 day after plating ookinetes. Oocyst media were composed of either RPMI 1640- and/or Schneider-based medium, with supplements including 15% v/v FBS, 7.5% v/v human serum (A+, pooled, heat-inactivated, Valley Biomedical), 10% v/v lipoprotein cholesterol concentrate (IC19147625, MP Biomedicals), 0.25% w/v trehalose (309,870,250, Thermo Fisher Scientific), 0.05% para-aminobenzoic acid (A9878, Sigma-Aldrich, MO), 0.04% w/v NaHCO₃ (BDH9280, VWR, US), 5 µg/ml silkworm haemolymph, 4% v/v human RBC lysate, 5 µM haemin chloride (3741Sigma-Aldrich, MO), 10% v/v fatty acid supplement (F7050, Sigma-Aldrich, MO), and 1% v/v Penicillin/Streptomycin solution (15,140,122, Gibco, US). The respective compositions of the media are described in Table 1.

Silkworm haemolymph collection

Bombyx mori silkworms, which originated from The Silkworm Shop (San Diego, CA), were fed with prepared and ready-to-eat fresh white mulberry-based food (Silkworm

Table 1 Oocyst medium compositions

Reagents	Medium			
	A	B	C	D
RPMI 1640	+	–	+ ^a	+ ^a
Schneider	–	+	+ ^a	+ ^a
FBS (15% v/v)	+	+	+	+
Lipoprotein, cholesterol (10% v/v)	+	–	+	+
Human serum (7.5% v/v)	–	–	–	+
Trehalose (0.25% w/v)	+	–	+	+
Para-aminobenzoic acid (0.05% v/v)	–	+	–	–
Silkworm haemolymph (5 µg/ml)	–	–	+	+
Human red blood cell lysate (4% v/v)	–	–	+	+
Hemin chloride (5 µM)	–	–	+	+
Fatty acid supplement (10% v/v)	–	–	–	+
NaHCO ₃ (0.04% w/v)	+	+	+	+
Penicillin/Streptomycin (1% v/v)	+	+	+	+

^a RPMI/Schneider in a ratio 1:1

Shop, San Diego, CA) at 25 °C until they developed into fifth-instar larvae or the prepupal stage (6–8 cm, 1.5–2"). Large and live silkworms were sprayed with cold PBS and dried with Kimwipes. Each silkworm larva was transferred to a 50 ml conical tube and placed on ice for a few minutes. Haemolymph (0.05 to 0.1 mL) and fat bodies of all silkworms were harvested by clipping the sides of two or three lower pairs of abdominal legs. During collection, the silkworms were euthanized by beheading. The collected haemolymph was pooled and heat-treated at 60 °C for 30 min and then chilled and centrifuged at 15,000×g for 30 min. Finally, the absorbance at 280 nm was measured to determine the approximate protein concentration. Haemolymph was aliquoted to avoid repeated thaw/freeze steps and stored at – 20 °C for long-term storage.

In vitro oocyst culture in a 3D alginate sponge

An AlgiMatrix sponge 3D culture system (known as an alginate sponge, A1098201, Thermo Fisher) was conditioned in a 6-well plate and used as recommended by the manufacturer. Ookinetes were purified by LS MACS columns as described above and mixed with 2 ml of AlgiMatrix firming buffer [composed of 1 part firming buffer and 9 parts oocyst medium C mixed with 50 µg/ml collagen IV and 25 µg/ml laminin/entactin (ECL, Corning NY)]. This firming buffer was added to each well of a 6-well plate. Approximately 750,000 ookinetes were seeded per well, except in one well, which was used as a negative control well. The 6-well plate was then incubated at 27 ± 2 °C for 2 h to allow absorption of ookinetes into the alginate sponge, followed by the addition of another 2 ml of oocyst medium C to each well. The medium was changed every 5 days, and the supernatant was collected.

In vitro sporozoite collection from the alginate sponge

The in vitro-produced sporozoites were collected by harvesting the supernatant from each well of the alginate culture on days 10, 15, 20, and 25. All supernatants from experimental wells, including the SPZ, were combined, whereas those from the control well were treated separately. The supernatants were centrifuged at 15,000×g for 10 min at 4 °C. The pellet was suspended in PBS, and sporozoites were counted with a haemocytometer after the cells had rested on the slide for 10 min. Production of in vitro sporozoites was performed in biological triplicate.

Immunofluorescence assays (IFA) of mosquito midgut oocysts

Plasmodium falciparum NF54HT-GFP-Luc-infected mosquito midguts were dissected on day 8 after gametocyte infection as described previously [22]. Briefly, the midguts were washed in cold PBS and fixed with blocking buffer composed of 4% v/v paraformaldehyde (PFA) in PBS overnight at 4 °C. The midguts were then permeabilized with 0.05% v/v Triton-X-100 solution (A16046, Thermo Fisher, USA) in blocking buffer for 30 min at RT, washed in cold PBS, and incubated again in blocking buffer for 1 h at RT. After being washed thoroughly, the oocysts were identified in mosquito midguts as GFP-expressing fluorescent reporter strains.

Detection of oocysts by immunofluorescence assays (IFA)

To detect oocysts seeded in the 2D culture system (i.e., 8-well chamber slides coated with ECL), the medium was aspirated, and a solution of 4% v/v PFA was added to each well for 30 min at RT for fixation. IFAs were performed on specific days of the oocyst growth cycle (days 5, 11, 15, and 20, as shown in Fig. 2). Each well was washed with PBS and blocked with blocking buffer (3% w/v BSA in PBS) (2630, Sigma) for one hour before being incubated with primary anti-PfCSP rabbit polyclonal antibodies (1:300 in blocking buffer) (Pocono Rabbit Farm) or anti-PfCap380 rabbit polyclonal antibodies [23] (1:250 in blocking buffer) for one hour at RT. The wells were then washed three times with PBS and blocked in blocking buffer for one hour at RT. A secondary antibody (Alexa Fluor 594-conjugated goat anti-mouse, A-11005; Invitrogen, 1:1000) was added, and the samples were incubated for 45 min at RT. The wells were washed with PBS, and the chamber slides were removed and allowed to air-dry. ProLong Diamond Antifade Reagent (P36966, Life Technologies) containing DAPI was applied, and the slide was sealed with nail varnish.

Oocysts were also identified by their GFP expression within the 2D culture system. On days 7, 15 and 25 of

culture, GFP-expressing oocysts were manually counted in 100 random microscopic fields of view (objective 40×) and classified depending on their size, as displayed in Fig. 1B. see Fig. 2

To visualize the GFP-expressing oocysts growing inside the 3D culture system (days 5, 10, 15, 20, and 25, as shown in Fig. 3C), one alginate sponge was used per

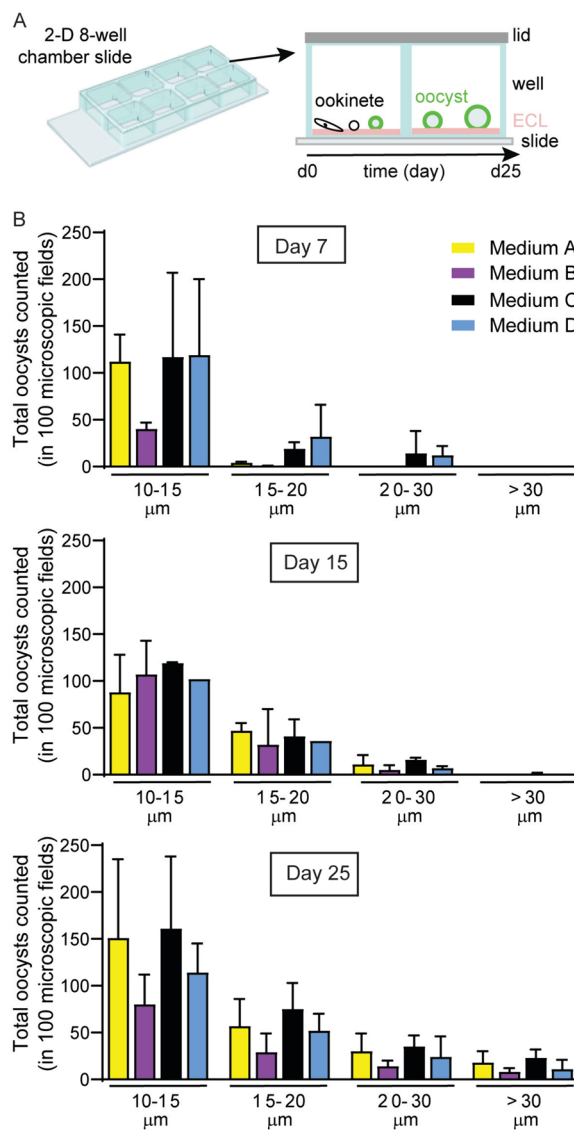


Fig. 1. 2D culture system for evaluating oocyst medium compositions. **A** Schematic of the 2D 8-well culture chamber coated with collagen, laminin, and entactin (ECL), on which 20,000 purified ookinetes (depicted in black) were seeded and allowed to grow into oocysts (shown in green) from day 0 to day 25. **B** On days 7, 15 and 25, while growing in the four different media, the PNF54-GFP-expressing oocysts were counted in 100 different microscopic fields of view, and their sizes were classified from 5 μm to 30 μm. Each medium is represented by a colored bar as indicated in the figure

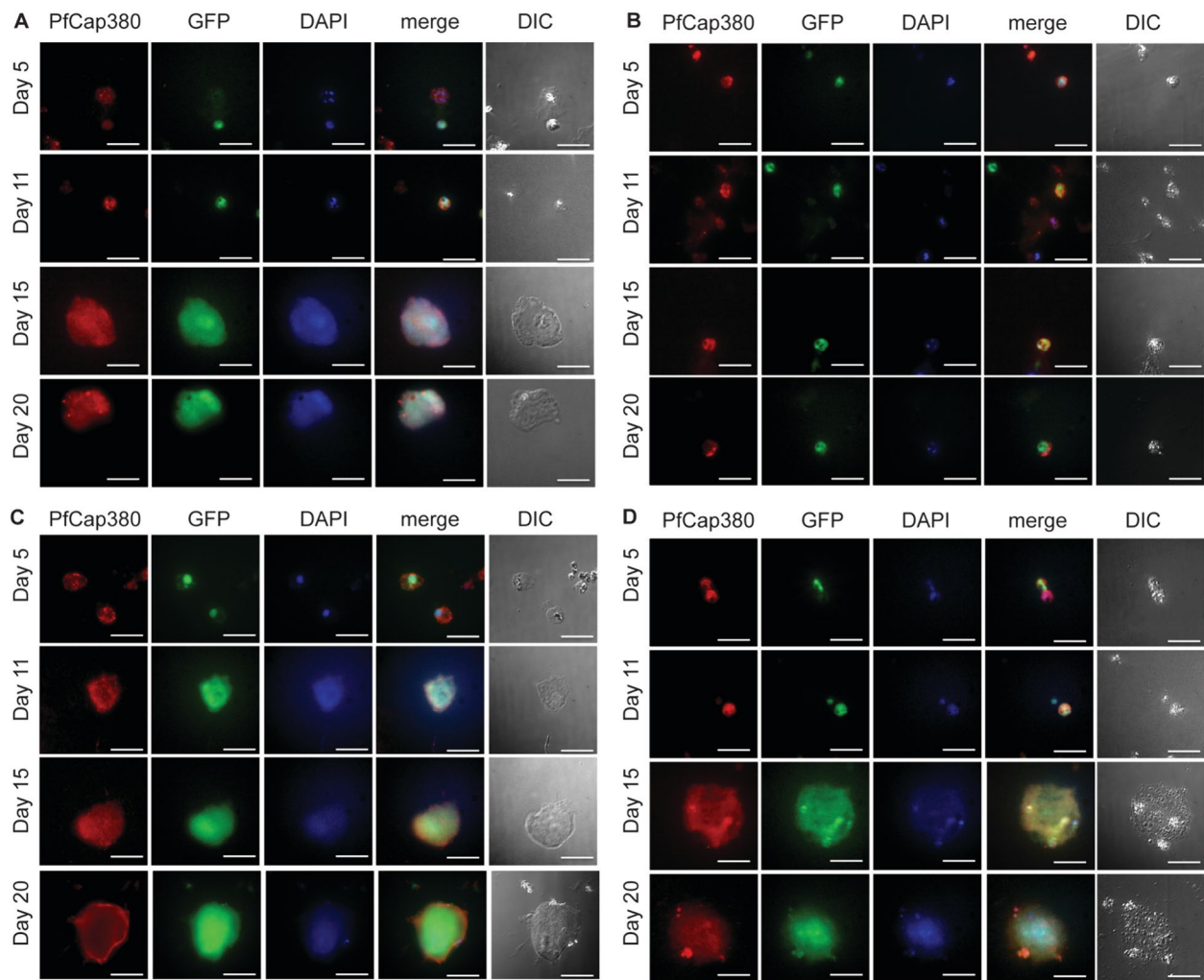


Fig. 2 Oocyst development in different culture media. **A–D** Nuclear division and oocyst growth in media A (**A**), B (**B**), C (**C**) and D (**D**) on days 5, 11, 15, and 20 are shown. PfNF54-GFP-expressing oocysts were stained with anti-oocyst capsule PfCap380 antibody and DAPI for their nuclei. Merged pictures and differential interference contrast (DIC) are indicated. The scale bar is 10 μ m

well. Each alginate sponge was cut into four equivalent portions, and one quarter of each portion was analysed by IFA. For this purpose, the alginate sample was placed on a well of a 6-well plate and washed three times with PBS. After fixation with 4% v/v PFA in PBS for 10 min at RT, the alginate samples were washed with washing buffer (0.02% w/v BSA in PBS) three times at RT and then permeabilized using a 0.01% v/v Triton-X-100 solution (A16046, Thermo Fisher, US) in PBS for 15 min at RT. The alginate samples were washed again three times with washing buffer and then incubated in blocking buffer (1% w/v BSA, 5% v/v FBS in PBS) for 15 min at RT. After being washed thoroughly, the oocysts were identified in mosquito midguts as GFP-expressing fluorescent reporter strains.

In vitro sporozoite immunofluorescence assay (IFA)

Sporozoites were harvested as described above and added to an 8-well chamber slide that was precoated with anti-CSP antibodies. Sporozoites were allowed to adhere to the slides for 30 min before being fixed with 4% v/v PFA in PBS for 30 min at RT. After washing with PBS, the slides were blocked with blocking buffer (3% w/v BSA in PBS) for one hour prior to incubation with a primary antibody against PfCSP (1:300) for one hour at RT. The samples were then washed three times with PBS and blocked again as described above. The membrane was then incubated with Alexa Fluor 594-conjugated goat anti-mouse secondary antibody (1:1000) for 45 min at RT before being washed three times, and the chamber was removed. ProLong Diamond Antifade Reagent (P36966,

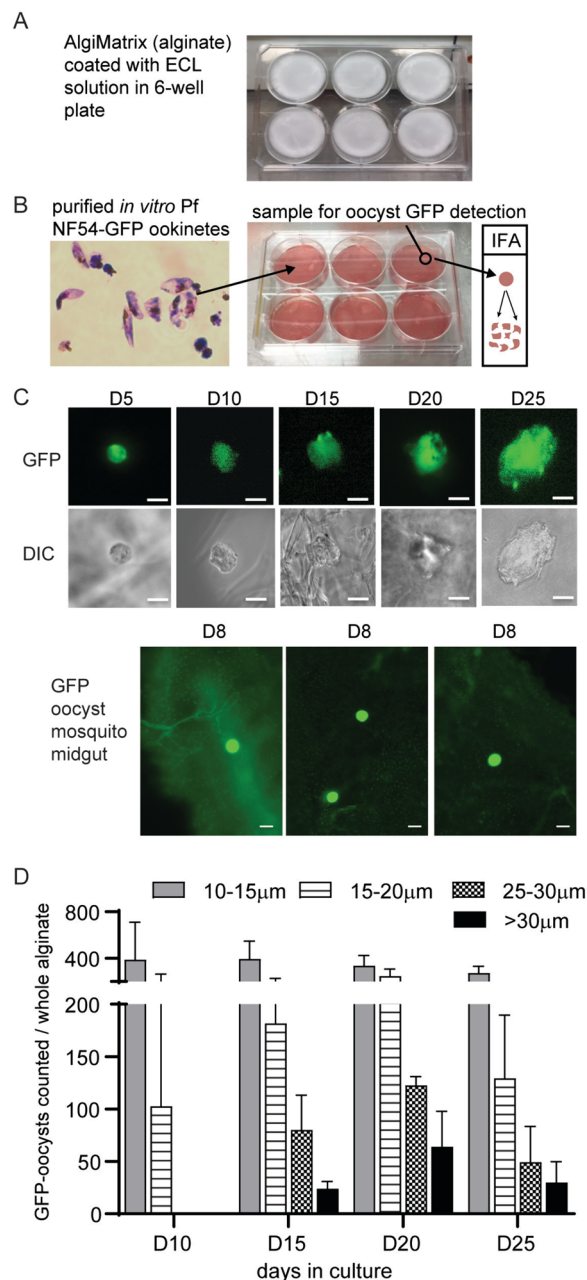


Fig. 3 Alginate sponge as a 3D culture system for oocyst development. **A** AlgiMatrix (alginate sponge) conditioned in a 6-well plate is coated with ECL matrix. **B** Purified PfNF54 GFP ookinetes are seeded into alginate sponge on day 0. To detect GFP-expressing oocysts, a small fragment of alginate is punched out and spread on a glass slide. **C** Pictures of GFP-expressing oocysts on different days of culture in the alginate are shown. The scale bar is 5 µm. The bottom panel shows GFP-expressing oocysts isolated from the mosquito midgut on day 8. The scale bar is 20 µm. **D** On days 10, 15, 20, and 25, the number of GFP-expressing oocysts was reported based on the estimated size (in micrometres)

Life Technologies) containing DAPI was added to each well prior to placing a coverslip that was sealed with nail varnish.

Microscopy

All IFA images were captured using a Delta Vision Elite High Resolution Microscope (GE Healthcare Life Sciences, PA) designed for fluorescence imaging and analysed using Delta Vision software (SoftWoRx software version 6.5.2). Images were taken at either 40× or 100× magnification with a 10× eyepiece. Images of the oocysts in the alginate sponge were captured, and the oocysts were counted through an upright fluorescence microscope (Nikon, Eclipse E600).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 10.2 (GraphPad, San Diego, CA, USA). A value of $p < 0.05$ was considered significant. The specific tests employed are indicated in the figure legend.

Results

Plasmodium falciparum oocysts need nutritional supplements for their development

Although gametocyte transformation into ookinetes can be achieved via *in vitro* *P. falciparum* culture [19], growing ookinetes that can develop into oocyst-producing sporozoites *in vitro* is a challenge. The initial transformation of ookinetes into oocysts was previously demonstrated [22], but their growth arrested prematurely by day 6, possibly due to nutritional deficiency in the original oocyst medium. To optimize the growth medium, four media (Table 1A–D) were evaluated in a 2D culture system (Fig. 1A) (8-well chamber slides precoated with ECL composed of 50 µg/ml collagen IV and 25 µg/ml laminin/entactin).

Purified PfNF54HT-GFP-luc ookinetes were seeded at 20,000 per well. The medium was changed every 2 days. This 2D culture allows for the attachment of ookinetes, followed by differentiated oocyst growth up to 25 days post-seeding (Fig. 1A).

Oocyst development was evaluated by two independent immunofluorescence (IFA) assays. In the first IFA, development and nuclear division were evaluated from day 5 to day 20 after incubation in each of the four media (Fig. 2A: Medium A; Fig. 2B: Medium B; Fig. 2C: Medium C; Fig. 2D: Medium D). To evaluate their development, the GFP-expressing oocysts were also stained with anti-PfCap380 antibodies to detect their specific capsule protein Cap380 [22] and with DAPI to detect their nuclei (Fig. 2).

The second IFA was used to count the *P. falciparum* oocyst-expressing GFP (PfNF54-HT-GFP-Luc) oocysts

in 100 random microscopic fields of view on days 7, 15 and 25 (Fig. 1B). Their diameter was visually classified into 4 ranges: 5–10 μm , 15–20 μm , 20–30 μm , or greater than 30 μm (Fig. 1B). Overall, most of the oocysts grown in medium A (RPMI 1640-based) developed slowly, remaining small ($\sim 5 \mu\text{m}$) on day 15 (Fig. 2A), and none developed beyond that date (Fig. 1B, yellow bar graph). Replacing the RPMI-based medium with Schneider-based medium in medium B did not favour oocyst growth (Fig. 1B, 2B, purple bar graph). Neither a restricted RPMI 1640-based medium (Medium A) nor a restricted Schneider's-based medium (Medium B), supplemented with FBS, was sufficient to provide nutrients for oocyst growth and maturation beyond the first few days. Medium C was then created by mixing RPMI-1640 and Schneider in a 1:1 ratio and supplementing with human blood lysate, haemin chloride, and silkworm haemolymph. Oocysts in Medium C grew larger from day 5 onward and reached a maximum size of 20–30 μm in diameter (Fig. 2C) by days 20–25 (Fig. 1B, black bar graph). Another medium was tested, Medium D, composed of Medium C supplemented with 7.5% human serum and 10% fatty acid supplement, which promoted the initial growth of the oocysts up to day 10 (Fig. 2D). However, their size and number decreased in culture for 15 days, indicating stalled metabolism (Fig. 1B, blue bar graph).

Medium C, an RPMI-Schneider-based medium supplemented with trehalose, silkworm hemolymph, human blood lysate, and hemin chloride, was found to be the best culture medium for overall consistent growth, and maturation of the oocyst up to 30 microns in diameter by day 25 (Figs. 1B and 2C). Compared to the results of a few studies [23–25] describing the size of midgut-derived oocyst as above 10 μm by day 8 or 9, the in vitro oocysts grown in medium C were found by day 11 most closely resembled to spherical in vivo oocysts. Indeed, from Fig. 2 on day 11, the oocysts' size in medium A was calculated to be about $\sim 5 \mu\text{m}$ in diameter, in medium B $\sim 5 \mu\text{m}$, in medium C $\sim 13 \mu\text{m}$, and in medium D $\sim 5 \mu\text{m}$. Hence, medium C was chosen for all further experiments.

Alginate sponges provide an enhanced 3D environment for oocyst development

To provide ookinetes with an improved 3D environment for better attachment and growth, we evaluated the use of the AlgiMatrix 3D culture system (referred to as an alginate sponge) for oocyst maturation and possible in vitro sporozoite production. The alginate sponge is a scaffold free of materials originating in animals that facilitates 3D cell culture with a pore size of ~ 50 –200 μm . Its nontoxic and macroporous structure is manufactured using pharmaceutical-grade materials that are stable at room temperature. It enables improved cell loading and

excellent nutrient delivery to cells [26, 27]. The alginate sponge was coated with extracellular matrix-like type IV collagen, entactin, and laminin (ECL) to mimic the midgut environment and provide a 3D anchoring matrix for attachment and maturation of growing oocysts (Fig. 3A). Fourteen days after gametocyte culture induction, exflagellation of GFP-expressing *P. falciparum* NF54 mature gametocytes was assessed, and zygote formation was induced for ookinete development. The next day, purified PfNF54 GFP-Luc ookinetes (Fig. 3B) were seeded into an alginate sponge (500,000–750,000/well) (seeding day is designated as day 0 of the oocyst growth cycle) and allowed to grow for up to 25 days post-seeding. Once the ookinetes attached to the ECL on day 0, they began to differentiate into oocysts. Using the PfNF54 GFP-Luc strain, the mature GFP-expressing oocysts were visualized by microscopy by punching out random small fragments of the alginate matrix and mounting them between a slide and coverslip (Fig. 3B). Oocysts attachment to the alginate sponge matrix and their growth were monitored starting on day 5 (approximately 5 μm in diameter) and continuing until day 25 post-seeding (approximately 30 μm in diameter) (Fig. 3C). The in vitro-generated oocysts were compared to GFP-expressing oocysts purified from mosquito midguts on day 8 (Fig. 3C, bottom panel) and they exhibited similar spherical shapes and sizes, about 10 μm between days 5 and 10 (Fig. 3C, top panel).

The GFP-expressing oocysts were counted over the course of their 25-day growth. Over time, many oocysts remained small (~ 10 –15 μm) for up to 25 days in culture, suggesting aborted maturation (Fig. 3D, gray bar graph). By day 20 of culture, approximately 15–20% of the oocysts had matured to 15–20 μm in size (Fig. 3D, striped bar graph), while another 15% continued to grow to 25–30 microns in size (Fig. 3D, dotted bar graph). Only a small portion ($\sim 5\%$) of the oocysts grew to a large diameter of 30 μm by day 20 or 25 (Fig. 3D, black bar graph). As anticipated, the in vitro development of oocysts is very challenging, but a few oocysts continued to mature beyond days 20–25 of their growth cycle, a critical development for in vitro sporozoite production.

The alginate sponge yields acceptable in vitro sporozoite production

Following the maturation of the oocyst within the alginate sponge, in vitro-produced sporozoites (IVS) were collected from the supernatant. Supernatants were collected on days 10, 15, 20, and 25, and then centrifuged, after which the IVS were counted on a haemocytometer after resting for 10 min. While the first few SPZs ($\sim 2567 \pm 809$ SPZ/alginate) were harvested as early as day 10 (Fig. 4A), IVS production increased by day 15

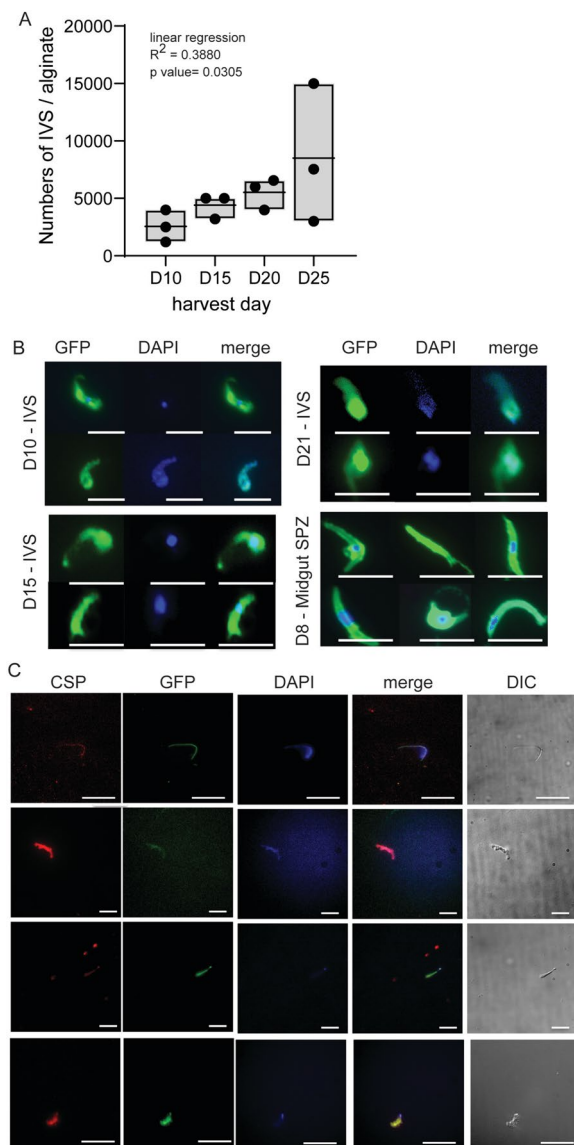


Fig. 4 Production of in vitro sporozoites (IVS) in alginate sponge. **A** IVS production per alginate sponge from days 10 to 25 following ookinete seeding. Floating bars show min to max with the mean. Linear regression was calculated with a R^2 value of 0.3880 and p value of 0.0305. **B** Morphology of GFP-expressing IVS on days 10, 15, and 21 post-ookinete seeding. Sporozoites dissected from the midgut on day 8 post-feeding were used for comparison. **C** IVS harvested on day 20 were stained with anti-CSP antibodies, DAPI for nuclei, and GFP expression. Merged IFA and differential interference contrast (DIC) pictures are shown. The scale bar is 10 μ m

($\sim 4400 \pm 600$ SPZ/alginate) and increased twofold by day 20 ($\sim 5517 \pm 775$ SPZ/alginate) (Fig. 4A). On day 25, the average IVS production was slightly greater than that on day 20, but there was more variation among the replicated experiments ($\sim 8517 \pm 3498$ SPZ/alginate) (Fig. 4A). Despite the limited sample size ($n=3$) and the data

scatter at day 25, resulting in a modest R^2 value of 0.3880, the upward trend in the IVS harvest yield from day 10 to 25 achieved significance according to linear regression ($p=0.0305$).

The morphology of the IVS was observed by IFA on days 10, 15, and 21 and compared to that of SPZ dissected and purified from mosquito midguts on day 8 (Fig. 4B). Characterized by their GFP expression pattern with DAPI staining of nuclei, IVS displayed the characteristic “deformed” shape of the midgut SPZ, rather than the elongated shape of the SPZ isolated from mosquito salivary glands (Fig. 4B). The haemolymph like-IVS morphology is distinctive from the mosquito derived SPZ, with a more pronounced motile sickle or spindle or hook shape. Those are similar to the deformed shape observed for the SPZ extracted from mosquito midgut oocysts on day 8 (Fig. 4B). The length of the IVS (around 10 μ m) was comparable to that of the midgut SPZ, but the haemolymph like-IVS seemed slightly rounded in one extremity and thicker (Fig. 4B). The midgut SPZ appear more elongated (Fig. 4B). CSP is known to be an essential protein for the midgut SPZ to escape from the oocyst in the midgut wall and move into the haemolymph. It is involved in mobility, migration, and invasion. Using anti-CSP antibodies on day 20, the haemolymph like-IVS expressed this essential protein on their surface (Fig. 4C).

Together, these findings demonstrate that this new 3D platform yields in vitro SPZs, although the numbers are modest and the SPZ emerge later (days 15–25) than in vivo midgut SPZ (estimated days 7–9). Thus, additional experiments are needed to improve the yield and the timing of release of IVS and characterize their infectivity.

Discussion

Production of in vitro sporozoites (IVS) of *Plasmodium* parasites without involvement of mosquitoes would render whole-organism vaccines far more practical, and would also contribute to the progress of research on malaria. Here, was presented a culture platform that includes a 3D matrix to provide an attachment substrate for *P. falciparum* ookinetes, allowing the growth of mature oocysts. In the past, to mimic the mosquito midgut in vitro and study ookinete invasion and oocyst maturation, a murine basement membrane-like gel (Matrigel) cocultured with *Drosophila melanogaster* cells (Schneider’s L2) was employed by many researchers [17, 28]. However, the use of insect cells in these procedures presents significant obstacles to use in humans. The two new culture systems described herein enable oocyst maturation and SPZ production without insect cells. The first 2D-culture system presented with the ECL coated on an 8-well chamber slide allowed us to visualize

the growth of the oocyst with various growth media. This led us to define the optimum growth medium for oocyst-stage maturation (Medium C). This material included trehalose, silkworm haemolymph, human blood lysate, and hemin chloride as essential components. Although this 2D culture model simulates the spatial midgut epithelium environment for the ookinete to mature into an early oocyst stage, it did not lead to production of in vitro SPZ. Indeed, within the mosquito, after passing through the midgut epithelial, the ookinetes lodge between the basal lamina and the epithelial cell plasma membrane where they start dividing into five stages, but only a fraction (approximately 0.1%) of the total ookinetes fully mature into oocysts [19, 29]. To overcome this challenge, the use of the 3D AlgiMatrix platform was optimized to mimic ookinete migration through the midgut epithelium, followed by maturation into SPZ-producing oocysts. The main advantage of this 3D culture system is its animal origin-free bioscaffold that facilitates 3D cell culture (25,26). The alginate sponge is composed of pharmaceutical-grade materials with a nontoxic structure, leading to improved cell attachment and nutrient delivery while ensuring cell integrity (25,26). The in vitro oocysts cultured within this sponge were able to complete their maturation (reaching over 30 μm in size) up to the release of IVS, increasing the interdisciplinary nature of these 3D cell culture models. The in vitro produced oocysts were similar in size to those derived from mosquito midgut. Musiime et al. [24] described the *P. falciparum* oocyst midgut as approximately 7 μm at days 2–3 and up to 30–40 μm by days 9–10. However, maturation of the in vitro oocysts seems to take slightly longer than in vivo in the mosquito midgut. Indeed, midgut oocysts can mature by day 9 or 10 [24], whereas the in vitro oocyst reaches this size range by day 15 to 25. An enhanced experimental design is currently under development to support faster oocyst maturation and thus early release of the IVS.

The IVS produced in these systems were thicker, but long and curved, and expressed CSP by 20 days in culture, thus resembling the development and morphology of SPZ found in mosquito. However, the timing of the release of SPZ from the oocysts is later than in the natural mosquito life cycle of midgut-SPZ production, in which release occurs around days 8–9. Extensive work is still needed to optimize the quantity, quality, consistency, and timing of release of these IVS. Future projects will include characterizing gene expression and immunogenicity in an in vivo FRGhuHep mouse model with humanized livers.

Conclusions

An optimum growth medium to complete oocyst maturation using the alginate sponge was determined that included trehalose, silkworm haemolymph, human blood lysate, and haemin chloride as essential components. This 3D culture system with AlgiMatrix provides adequate adhesion and supports the full growth of *P. falciparum* oocysts up to the release of in vitro sporozoites.

Abbreviations

<i>P. falciparum</i>	<i>Plasmodium falciparum</i>
WHO	World Health Organization
SPZ	Sporozoite
IVS	In vitro sporozoite
WO	Whole organism
GAP	Genetically attenuated parasite
GFP	Green fluorescent protein
LS MACS	Labeling systems Magnetic column cell separation
RBC	Red blood cells
FBS	Fetal bovine serum
RPMI	Roswell Park Memorial Institute
RT	Room temperature
IFA	Immunofluorescence assays
$^{\circ}\text{C}$	$^{\circ}\text{Celsius}$
PBS	Phosphate-Buffered Saline
ECL	Entactin collagen laminin
BSA	Bovine serum albumin
CSP	Circumsporozoite protein
PFA	Paraformaldehyde
FRGhuHep	Fah, Rag2, and Il2rg mutations in humanized hepatocytes mouse

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Author contributions

YZ, AKG, and MA conceived and designed the experiments; YZ, KH, ZM, ML, JWD and AMG performed the experiments; YZ, LSI, JWD, ZM, AKG and MA analyzed and interpreted the data; YZ, AKG, and MA drafted the manuscript. All authors (except AKG who passed away) have read and approved the final manuscript.

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Availability of data and materials

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable. No personal identifiable data was used in the study. All authors have provided consent to publish the findings in this study.

Competing interests

The authors declare no competing interests.

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