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In Vitro Generation of *Plasmodium falciparum* Ookinetes

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Abstract. *Plasmodium* transmission from the human host to the mosquito depends on the ability of gametocytes to differentiate into ookinetes, the invasive form of the parasite that invades and establishes infection in the mosquito midgut. The biology of *P. falciparum* ookinetes is poorly understood, because sufficient quantities of this stage of this parasite species have not been obtained for detailed study. This report details methods to optimize production of *P. falciparum* sexual stage parasites, including ookinetes. Flow cytometric sorting was used to separate diploid/tetraploid zygotes and ookinetes from haploid gametetocytes and unfertilized gametes based on DNA content. Consistent production of 10^6–10^7 *P. falciparum* ookinetes per 10 mL culture was observed, with ookinete transformation present in 10–40% of all parasite forms. Transmission electron micrographs of cultured parasites confirmed ookinete development.

INTRODUCTION

Malaria affects more than 500 million people and kills an estimated 900,000 people each year.[1] Interest in the potential of transmission-blocking vaccines to control or even eradicate malaria has recently increased; such vaccines work by inducing antibodies against components of the parasite's sexual stage forms, hence reducing infectivity of the reservoir, humans, for the obligate vector, *Anopheles* mosquitoes.[2–11] More detailed understanding of *Plasmodium falciparum* transmission stage biology, particularly ookinetes, would directly contribute to human transmission-blocking vaccine development.

Plasmodium sexual development occurs in the mosquito midgut. Mature gametocytes taken up with the mosquito blood meal emerge from erythrocytes as gametes. Male microgametes fertilize female macrogametes to generate zygotes. The parasite transforms into a motile, constitutively secreting and invasive ookinete at the same time that it is undergoing genetic recombination.[12–16] Sexual stage-specific antigens are potential targets for transmission-blocking antibodies, which has been most robustly shown in animal models of malaria.[16,17] Because of experimental challenges, the biology of few sexual stage antigens of *P. falciparum* is understood in any detail.[18] Recent successes have been achieved in generating *P. falciparum* sexual stage parasites *in vitro*, including a recent study by Ghosh and others.[19,20] However, a detailed study of the biology of transmission-blocking antigens is still limited by the inability to generate large quantities of *P. falciparum* sexual stage parasites, particularly ookinetes, *in vitro*.

Here, we report optimization of methods that allow for the consistent generation of 10^5–10^6 *P. falciparum* ookinetes per 10 mL *in vitro* culture.[19,21–25] Flow cytometry sorting based on detection of DNA content allowed for the enrichment of different sexual state parasite forms that allow for the direct study of the different stages of *P. falciparum* sexual stage parasites.

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HIGH-YIELD OOKINETE PRODUCTION

The *P. falciparum* strain NF54 was maintained in continuous asexual culture according to standard protocol.[26] Human blood used for *in vitro* culture was freshly drawn from volunteers after informed consent according to a protocol approved by the University of California, San Diego Human Subjects Protection Program. Gametocytes were cultured as previously described[27] (Appendix), and the overall procedure is schematized in Figure 1. Morphologically mature microgametocytes were seen as early as 12 days for microgametocytes and 14 days macrogametocytes and as late as 22 days for both. To compensate for this lack of synchronization, gametocyte cultures were started 2–3 days apart to ensure that cultures containing mature microgametocytes could be mixed with cultures containing mature macrogametocytes.[26–29]

On days 14–17 of culture, mature microgametocytes were tested for the ability to exflagellate and emerge under standard conditions (Appendix).[30] Two gametocyte cultures with microgametocytes confirmed to be functional (≥ 4 exflagellation centers per 40× field) were combined with two gametocyte cultures with emergence-competent macrogametocytes (≥ 80% macrogamete emergence). The combined parasites were centrifuged, and the pellet was resuspended in heat-inactivated AB+ human serum to 10–20% hematocrit at 19–23°C (ambient laboratory temperature) for 30 minutes for gamete maturation and zygote fertilization. Sexual stage parasites were centrifuged, and the pellet was resuspended in freshly prepared filter-sterilized ookinete medium (RPMI–1640, 25 mM Hepes, 2 mM L-glutamine, 2 g/L $NaHCO_3$, 50 mg/L hypoxanthine, 15% heat-inactivated AB+ human serum or heat-inactivated fetal bovine serum [FBS], pH 8.2–8.4, with NaOH) to 10–20% hematocrit. Centrifugation steps were done at 19–23°C and 800 × *g* without brake. Parasites in ookinete medium were transferred in 10-mL aliquots to 25-cm^2 flasks and gently rocked at 19–23°C for 36–48 hours, although ookinetes could be seen in culture for up to 72 hours in ookinete medium.

The generation of large quantities of *P. falciparum* ookinetes was dependent on the presence of mature micro- and macrogametes, which, in turn, was dependent on the production of mature micro- and macrogametocytes (Figure 2). Gametocyte cultures contained up to 8.5% gametocytemia, and 50–90% of these gametocytes were stage V gametocytes (Table 1). Optimized cultures had an average ookinete density of 25%, with a yield of 5–50 × 10^6 ookinetes per 10 mL of ookinete

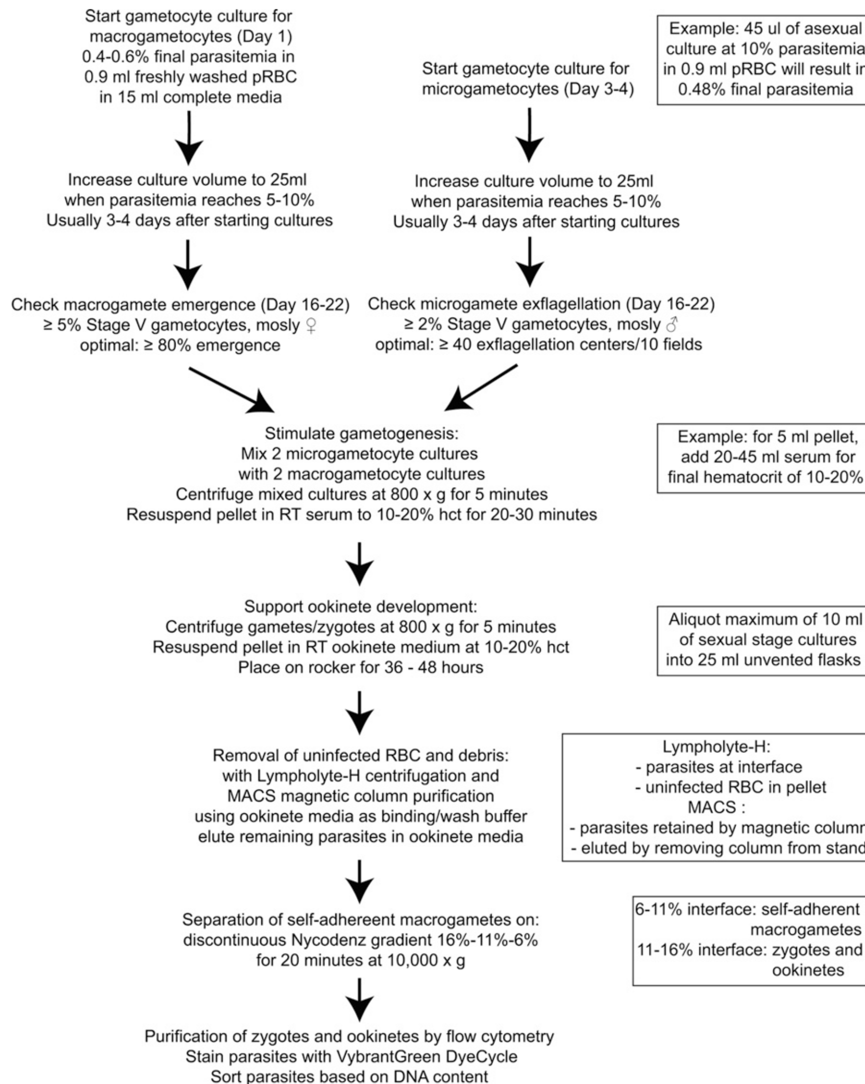

FIGURE 1. Flow chart of *P. falciparum* sexual stage parasite culture protocol. pRBC = packed red blood cells; ♂ = male; ♀ = female; Δ = heat inactivated; RT = room temperature (19–21°C); hct = hematocrit.

culture (Table 2). This yield is at least 4- to 8-fold better than previously described methods.[23,31]

PURIFICATION OF *P. FALCIPARUM* SEXUAL STAGE PARASITES

P. falciparum sexual stage cultures were produced as mixtures of uninfected red blood cells, asexual stage parasites, gametocytes, macrogametes, zygotes, and ookinetes. Stage-specific enrichment was approached using multiple purification methods, including single-step density centrifugation, magnetic separation, discontinuous density gradient centrifugation, and flow cytometry sorting. The majority of uninfected erythrocytes were removed from sexual stage parasite cultures by single-step density gradient centrifugation (Lympholyte-H; Cedarlane Laboratories, Burlington, NC) according to manufacturer's instructions. Parasites were collected from the gradient interface, washed two times in ookinete medium, and further purified by magnetic separation. Purification of parasites using density gradient removed approximately 90% of red blood cells. Further purification of parasites using magnetic separation resulted in removal of approximately 95–99% of red blood cells and facilitated sorting of parasites by flow cytometry. A MidiMACS magnetic separator with an LD-50 column was used to positively select for condensed hemozoin-containing gametocytes, macrogametes, zygotes, and ookinetes as well as any remaining asexual stage schizonts, according to manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany).[32] Magnetic purification of sexual stage parasite cultures was done at 19–23°C using ookinete medium instead of the manufacturer's recommended MACS® buffer. Magnet-retained sexual stage parasites were washed, eluted from the magnetic column, and then, centrifuged at 800 × *g*. The cell pellet was resuspended in 100–500 μL of ookinete culture medium and then placed on a 6–11% or 11–16% Nycodenz gradient (Sigma-Aldrich, St. Louis, MO).[30,33] Briefly, 400 μL of 6% Nycodenz, 400 μL of 11% Nycodenz, and 400 μL of 16% Nycodenz were layered in a 1.5-mL microcentrifuge tube; 200 μL of resuspended parasites were layered on top of the three-step gradient and centrifuged at 10,000 × *g* for 10 minutes at 4°C with no brake. The 6–11% contained self-adherent macrogametes, consistent with previous

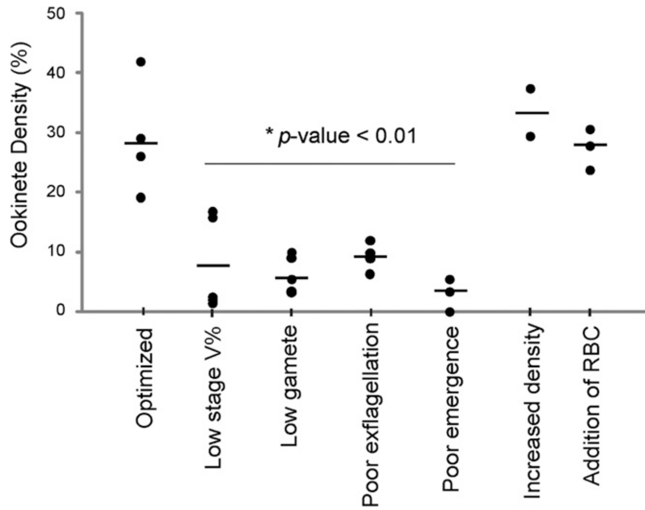

FIGURE 2. Gametocyte and gamete maturity significantly affect ookinete production. Ookinete density in sexual stage cultures depends on gametocytemia and gamete maturation. Sexual stage parasite cultures containing 19–42% ookinetes were consistently produced by mixing ≥ 5% macrogametocytemic cultures with ≥ 2% microgametocytemic cultures at a final hematocrit of 10–20%. Optimized = use of cultures with less than optimized parameters resulted in sexual stage cultures with ookinete densities of 3–17%. Low stage V%* = gametocyte cultures with less than 2% mature gametocytemia ookinetes. Low gamete* = cultures with low rates of both gamete emergence and exflagellation, defined as < 80% emergence and less than an average of four exflagellating centers per 40× field. Low exflagellation* = cultures with less than an average of four exflagellating centers per 40× field mixed with cultures exhibiting ≥ 80% macrogamete emergence. Low emergence* = cultures exhibiting ≥ 80% macrogamete emergence mixed with microgamete cultures with an average of four exflagellating centers per 40× field. Other factors that reportedly improved parasite transmission to mosquitoes, namely increasing parasite density and adding fresh drawn erythrocytes to the cultures, did not significantly improve ookinete development in vitro compared with other optimized conditions described here.[29] Increased density = increased density of sexual stage cultures by increasing final hematocrit from 10% to 20% (P value < 0.842). Addition of RBC = addition of erythrocytes drawn from volunteers and washed within 24 hours of sexual stage parasite cultures (P value = 0.489). Statistical significance was determined by analysis of variance (ANOVA). Asterisk indicates P value < 0.01. Of note, lack of a statistically significant difference may be caused by low sample numbers, particularly in the increased density and addition of RBC groups.

reports,[34] whereas the 11–16% interface contained ookinetes, zygotes, non–self-adherent macrogametes, and untransformed gametocytes.

Plasmodium zygotes and ookinetes are the only *Plasmodium* developmental stage cells known to be diploid/tetraploid.[35,36] This difference in ploidy was exploited to separate *P. falciparum* zygotes and ookinetes from other sexual stage forms using flow cytometric sorting. Parasites from the 11–16% Nycodenz gradient interface were washed and stained with Vybrant DyeCycle Green stain according to manufacturer's instructions (Molecular Probes; Invitrogen, Carlsbad, CA). Approximately 10^7–10^8 parasites were then passed through a 30-µm filter to remove clumps of self-adherent cells and were sorted by fluorescence intensity using a MoFlo high-speed sorter (Dako, Glostrup, Denmark). Stained parasites were analyzed by flow cytometry and divided into four subgroups based on fluorescence (Figure 3). Region R3 showed enrichment for gametocytes, and R4 showed enrichment for non-adherent macrogametes. The finding that macrogametes could be separated from gametocytes by DNA content was surprising but consistent with previous fluorometric studies.[37–41] Region R6 was enriched for a mixture of zygotes and ookinetes (Figure 3). The finding that the majority of zygotes and ookinetes had more than or equal to four times as much DNA as region R3 is consistent with our current understanding that meiotic division occurs shortly after gamete fusion.[36,37,42,43] It is possible that further refinements of flow cytometric sorting (for example, incorporation of forward- and side-scatter parameters to separate round forms from elongated forms based on size or clumping) might be able to separate zygotes from ookinetes.

TABLE 1
In vitro P. falciparum gametocyte production

Culture number	1	2	3	4	5
Asexual parasitemia	0.3%	0%	0%	0.4%	0.4%
Immature gametocytemia	3%	0.5%	2.2%	1%	2%
Stage V gametocytemia	5%	6%	6.3%	5%	5%
Total gametocyte yield	20×10^7	3.3×10^7	7.5×10^7	5×10^7	10×10^7

Gametocytemia of unpurified *P. falciparum* gametocyte cultures was determined when the majority of gametocytes were mature, as early as 16 days and as late as 22 days of culture; 1,100–2,400 cells were counted in 10 fields. Average yields were 2–20 × 10^7 gametocytes per 25 mL culture. Asexual stage-to-gametocyte transformation efficiencies for five representative gametocyte cultures used for ookinete preparations are shown.

TABLE 2
P. falciparum ookinete densities resulting from *in vitro* culture

Culture number	1	2	3	4	5
Gametocytes (%)	39%	45%	37%	32%	13%
Macrogametes (%)*	24%	16%	12%	31%	67%
Round forms (%)†	11%	9%	9%	11%	8%
Ookinetes (%)	26%	31%	42%	26%	12%
Ookinete yield	10×10^6	57×10^6	31×10^6	10×10^6	24×10^6

Purified sexual stage parasites were stained with Leukostat and examined by light microscopy to determine sexual stage parasite densities; 300–1,000 parasites in 10 fields were counted. Yields were 5–60 × 10^6 ookinetes per 10 mL culture. Transformation efficiencies for five representative cultures are shown.
* Self-adherent macrogametes.
† Other round forms including non–self-adherent macrogametes, zygotes, and retort ookinetes that could not be definitively distinguished from each other by light microscopy.

PFS25 AND CHITINASE (PFCHT1) DETECTION IN GAMETOCYTES AND OOKINETES

To see if Pfs25 or PfCHT1 could be used as markers to classify elongated parasites as gametocytes or ookinetes (Figure 4A and B), gametocytes and *in vitro*-generated ookinete cultures were examined by an immunofluorescence assay (IFA) (Figure 4C–H) using previously characterized antibodies to Pfs25[44] and chitinase,[45] two proteins presumed to be zygote/ookinete and ookinete-specific, respectively (Appendix).[46,47] By IFA, immunoglobulin G (IgG) negative control antibody did not generate fluorescence signal in either gametocytes or ookinetes, whereas antibodies to *P. falciparum* chitinase (PfCHT1) and Pfs25 produced fluorescent signals in gametocytes (as previously described for Pfs25)[48] and ookinetes (Figure 4C–H). The fluorescence signal was observed to be stronger in ookinetes than in gametocytes, with a distinct pattern consistent with surface localization of Pfs25 notable in ookinetes but not gametocytes.

Western immunoblots using the monoclonal antibody 1C3 to the *P. falciparum* chitinase PfCHT1 detected the protein in mixed gametocytes, untransformed gametes, and early zygotes

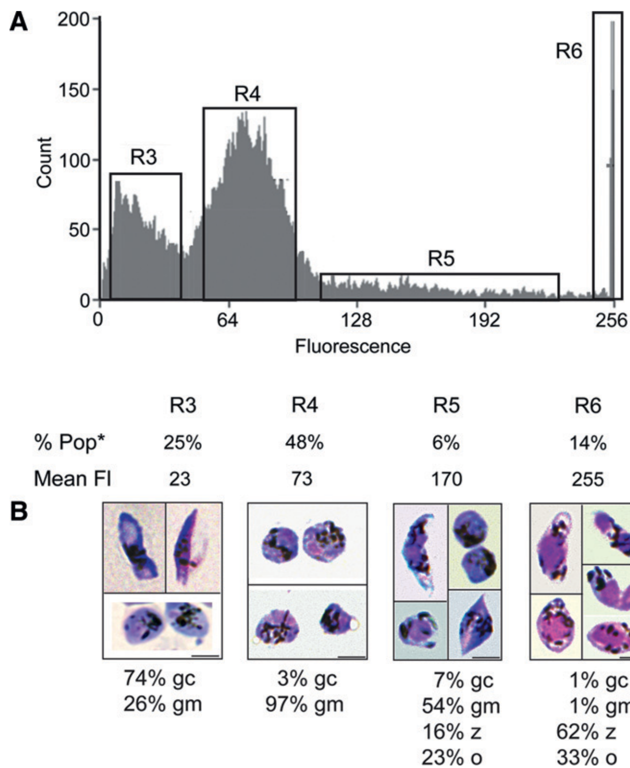

FIGURE 3. Flow cytometry sorting of *P. falciparum* sexual stage parasites, zygotes, and ookinetes based on DNA content. Flow cytometry-sorted samples were stained and analyzed by light microscopy to determine the dominant sexual stage form in each of the fluorescent regions populations identified by fluorescence intensity. (**A**) Vybrant Dyecycle Green-stained sexual stage parasites were analyzed by flow cytometry. Parasites showed differential fluorescence patterns divided into regions R3, R4, R5, and R6 based on fluorescence intensity. R3 and R4 constituted distinct fluorescent peaks. Percent of cells (% Pop) and mean fluorescence (Mean Fl) for each region are shown (*7% of the total cells counted are not contained within the four labeled gates shown and thus, could not be included in % Pop). (**B**) Sorted parasites from each population were stained with Leukostat and examined under light microscopy for quantification of parasite forms; 300–1,000 parasites were counted per sorted population. Gametocytes (gc) were the dominant form found in region R3; non–self-adherent round forms (gm) were found in region R4. Region R6 contained ookinetes (o) and round forms and likely contained retort ookinetes and zygotes (z), which expressed four times the fluorescence as regions R3 and R4 and represent gametocytes and likely, macrogametes. Less than 1% of parasite forms sorted into R3, and R4 was asexual stage parasite forms; 3–5% of all parasites sorted in R6 were asexual forms, mostly schizonts. (Scale bars: 5 μm.)

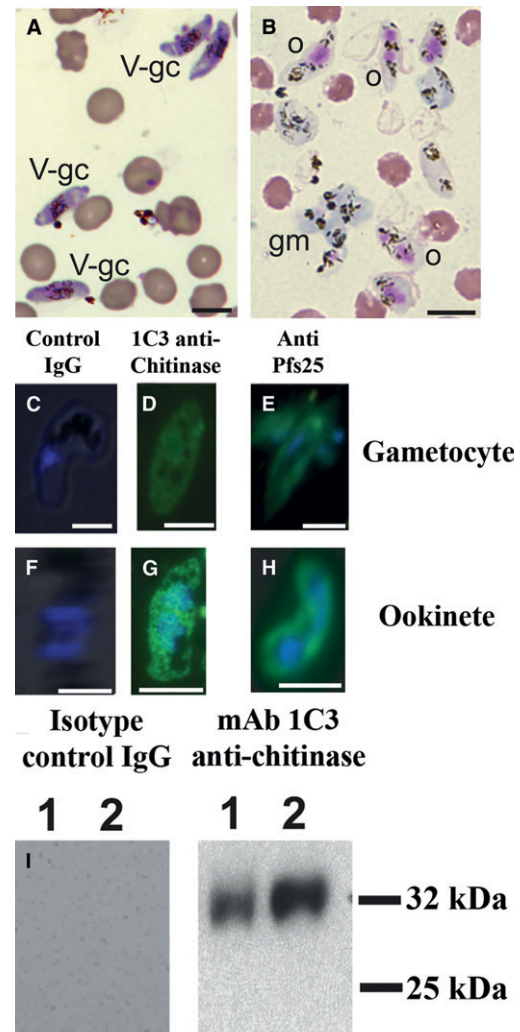

FIGURE 4. Light and fluorescence microscopy of *in vitro* cultured *P. falciparum* sexual stage parasites. (**A**) Leukostat-stained thin smears of unpurified gametocyte culture contained stage V gametocytes (V-gc). (**B**) Leukostat-stained thin smears of *in vitro* cultured *P. falciparum* sexual stage parasites. Ookinetes (o) were identified by the presence of one to two large eosinophilic nuclei and lack of a surrounding erythrocyte membrane. Round forms include macrogametes and zygotes. Self-adherent macrogametes (gm) were slightly basophilic and often found in clusters. Non-adherent macrogametes were not definitively distinguished from zygotes and retort ookinetes. Hemozoins appeared as dark brown pigment crystals. (**C–H**) Gametocytes from gametocyte cultures and ookinetes from sexual stage cultures were probed with antibodies to chitinase and Pfs25. DAPI (blue) was used to stain nuclear material; gametocytes contained one nucleus, whereas ookinetes contained one to two nuclei. (**C**) Gametocytes and (**F**) ookinetes probed with IgG isotype control antibody showed no reaction. (**D**) Gametocytes and (**G**) ookinetes probed with 1C3 monoclonal antibody against chitinase[45] (green) showed a diffuse, intracellular staining pattern in both gametocytes and ookinetes. (**E**) Gametocytes and (**H**) ookinetes probed with antibody against Pfs25[58] (green) showed an intracellular pattern in gametocytes and both intracellular and surface staining patterns with ookinetes. (**I**) Chitinase detected in gametocytes and a 72-hour cultured mixed zygotes and ookinetes sample by Western immunoblot. Equal numbers of cells gametocytes (lane 1) and untransformed gametes plus zygotes plus ookinetes (lane 2) were lysed in SDS, separated by SDS-PAGE, and probed with antibody against the *P. falciparum* chitinase PfCHT1 using monoclonal antibody 1C3.[45] IgG isotype control antibody did not produce a band in either sample. Antibody against PfCHT1 recognized an approximately 32-kDa band in both gametocyte and zygote plus ookinete samples and quantitatively more after 72 hours of cultures than in gametocytes.

directly and in 72-hour ookinete cultures (Figure 4I), with an increased intensity of PfCHT1 seen in mature ookinetes.

The quantitative and non-qualitative aspects of these finding indicate that neither the presence of chitinase nor Pfs25 protein, as detected by IFA or Western immunoblot, unequivocally distinguishes *P. falciparum* ookinetes from gametocytes.

ULTRASTRUCTURAL ANALYSIS OF *P. FALCIPARUM* SEXUAL STAGE PARASITES

Transmission electron microscopy (TEM) of sexual stage cultures was done to determine whether elongated, banana-shaped parasites were either gametocytes or ookinetes (Figure 5 and Appendix). Parasites submitted for TEM were

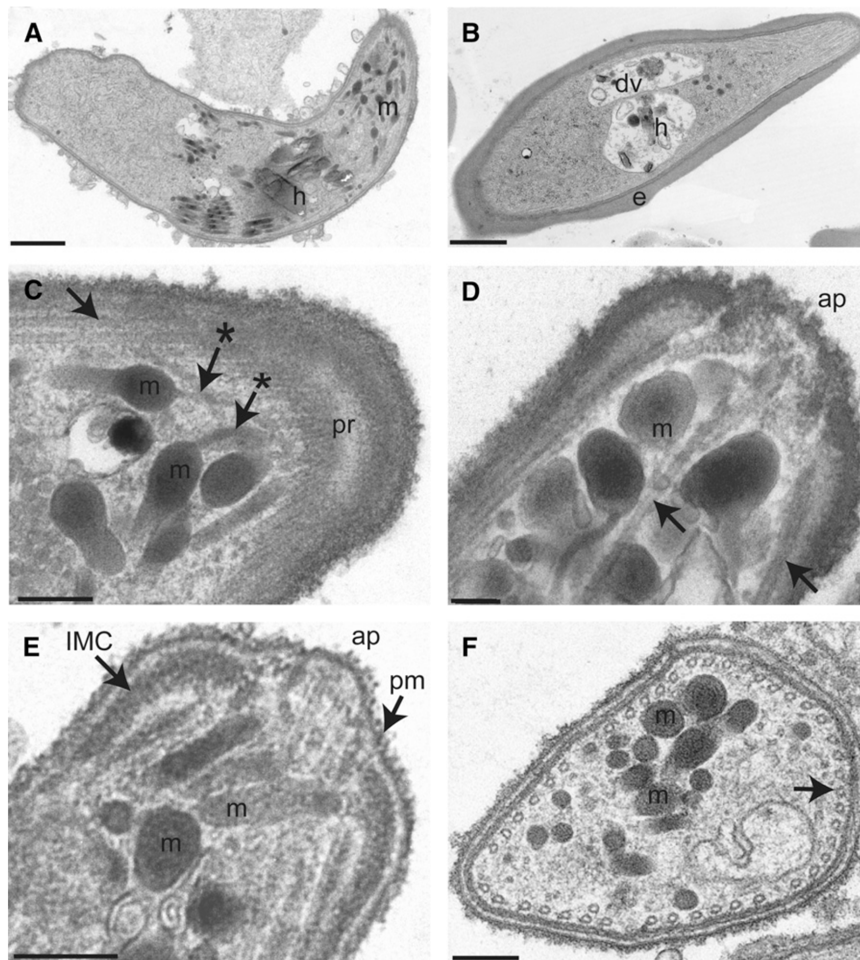

FIGURE 5. Ultrastructural features of the *P. falciparum* ookinete. (**A**) Sagittal section of an ookinete shows micronemes (m), seen as electron-dense round or cigar-shaped organelles, and a hemazoin crystal (h). (Scale bar: 1 μm.) (**B**) For comparison, a gametocyte within an infected erythrocyte (e) is seen with a discrete digestive vacuole (dv) with hemozoin (h). (Scale bar: 1 μm.) (**C**) Tangential section of the apical end of an ookinete showed microtubules (→), which converge at the polar ring (pr). This section showed narrow ducts of micronemes (m) that appear to track to the apical end (*→). (Scale bar: 200 nm.) (**D**) Transverse, midline section of an ookinete apical end showed the apical pore (ap), micronemes (m), and microtubules (→). (Scale bar: 200 nm.) (**E**) Transverse section of an ookinete showed micronemes (m) as well as the apical pore (ap) located between two electron-dense regions representing the inner membrane complex (IMC) underlying the plasma membrane (pm). (Scale bar: 200 nm.) (**F**) Transverse cross-section near the apical end of an ookinete shows micronemes (m) as well as microtubules (→) that circumferentially line the subpellicular space of the parasite. (Scale bar: 200 nm.)

enriched by magnetic and density gradient separation techniques. Approximately 30% of parasites were ookinetes as determined by light microscopic examination of Leukostat-stained slides. Qualitative analysis of cultivated parasites by TEM showed the presence of round parasite sections, which could have been cross-sections of macrogametes, zygotes, gametocytes, or ookinetes, as well as elongated parasite sections, which could have been gametocytes and ookinetes. Cross-sections of gametocytes and ookinetes could be identified by the presence of subpellicular microtubules, which supports the characteristic banana-shaped forms. Gametocytes had a surrounding erythrocyte membrane, whereas cross-sections of ookinetes did not.[41,49–55]

Examination of erythrocyte-free elongated parasites showed definitive ookinete ultrastructure, including the apical complex and a pellicle, which was not observed in gametocytes (Figure 5). The apical complex consists of an apical polar ring and micronemes. The polar ring serves to anchor the subpellicular microtubules.[55] Micronemes are round, protein-dense, membrane-bound organelles that have been shown to contain proteins secreted through their ducts near the apical end.[56,57] The ookinete pellicle includes the parasite plasma membrane, the inner membrane complex, and subpellicular microtubules.[35]

The method presented here consistently produced large quantities of sexual stage forms of the human malaria parasite *P. falciparum*. Currently, standard methods for consistently generating *P. falciparum* zygotes and ookinetes require feeding gametocytes to *Anopheles* mosquitoes followed by dissection of these sexual stage forms from mosquito midguts. Despite electron microscopic proof of *P. falciparum* ookinete production, a limitation of the present report is that the ability of *in vitro*-generated *P. falciparum* ookinetes to produce oocysts in mosquitoes was not verified. Nonetheless, this improved production of *P. falciparum* sexual stage parasites is a significant step to understanding biological details of ookinetes specific to this human malaria parasite. This work comes at a critical juncture as interest increases in malaria control

efforts based on the potential of *Plasmodium* transmission-blocking vaccines.

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Disclaimer: The authors declare no conflict of interest.

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APPENDIX: METHODS

***P. falciparum* gametocyte cultures.** The NF54 isolate of *P. falciparum* used in these experiments was a gift from Stephen Hoffmann (Sanaria, Rockville, MD). NF54 is one of the 13 initial Nijmegen *Falciparum* (NF) isolates derived from a Dutch patient.[52–54] This strain was maintained in continuous asexual culture according to standard protocol[26] with the exception that no antibiotics were used in the complete medium: RPMI 1,640, 25 mM Hepes, 2 mM L-glutamine, 2.4 g/L NaHCO3, 50 mg/L hypoxanthine, and 10% heat-inactivated AB+ human serum (Interstate Blood Bank, Memphis, TN). Asynchronous asexual stage cultures at 8–15% parasitemia were used to start gametocyte cultures. Gametocyte cultures were started by diluting asexual stage parasite cultures into 0.9 mL of freshly washed packed human red cells to generate a final concentration of 0.4–0.6% parasitemia in 15 mL total volume. Dilution of asexual cultures at 8–10% parasitemia generated gametocyte cultures with the highest yields. Spent gametocyte culture medium was removed and replaced daily with 15 mL of 37°C complete medium until cultures reached 5–10% parasitemia, usually by day 3–4.[29,55,56] At this point, spent gametocyte culture medium was replaced with 25 mL of 37°C complete medium for the remainder of the culture period. Additionally, approximately 10 mL of spent medium were left in the culture flask during each medium change.[57] Gametocyte cultures were maintained in a low oxygen environment by gassing the cultures with filtered 5% O_2, 5% CO_2, and 90% N_2.

Exflagellation and emergence assays. Mature macrogametocytes were tested for the ability to emerge using a modified exflagellation protocol: instead of examining slides for emergence in real time, blood smears were made 1 hour after gametogenesis, fixed with methanol, and stained with a modified Wright stain. The ratio of gametes to gametocytes was determined by counting thin smears 1 hour post-emergence.

Leukostat-stained light microscopy. A modified Wright stain using Leukostat dyes was used to stain parasite thin smears. Smears were stained for 15 seconds in Leukostat 1 (0.1% eosin Y, 0.4% Na_2HPO_4, and 0.1% formaldehyde), rinsed in distilled water, and stained for 30 seconds in Leukostat 2 (0.04% methylene blue, 0.04% Azure A, KH_2PO_4, and Na_2HPO_4). Slides were then rinsed in distilled water and left to air dry.

Immunofluroescence of cultured parasites. Cultured parasites were fixed and permeabilized on glass slides and probed with antibodies against chitinase (1C3), Pfs25 4B7 (MRA-28; deposited by David C. Kaslow, Malaria Research and Reference Reagent Resource Center, Manassas, VA), or

mouse IgG negative control as previously described[42] with fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary and 3 μM DAPI (Molecular Probes; Invitrogen, Carlsbad, CA).

TEM of cultured parasites. Sexual stage parasite samples were depleted of uninfected red blood cells and asexual parasite forms by centrifugation on Lympholyte-H single-step density gradient and washed three times with ookinete medium. Parasites were then centrifuged and resuspended in 500 L of ookinete medium. These cells were further prepared for TEM according to standard protocol.[58] Resulting parasite blocks were cut with a Reichert ultramicrotome, stained with 1% uranyl acetate and lead nitrate, examined using a JEOL 1200EX II transmission electron microscope (JEOL, Peabody, MA), and photographed using a Gatan digital camera (Gatan, Pleasanton, CA).