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To,
Dr Vineeta Bajaj
Review Editor
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Friday 04/24/2020

Dear Dr Bajaj,

Thank you for the opportunity to revise our manuscript "*Plasmodium falciparum* gametocyte culture and mosquito infection through artificial membrane feeding". We are grateful for careful review and very important constructive suggestions. We have revised the manuscript and accommodated most of the edits suggested by editorial team and reviewers. After revision process we believe manuscript has substantially improved.

Along with this letter, you will find editorial and reviewers' comments and our point by point response to each comment/suggestion. In order to accommodate most of the revision we needed significant assistant from two of our colleagues and they are now added as authors on the manuscript. During the review process we tracked changes and they are marked in the revised copy. All the co-authors have contributed and has given approval to the revised version of manuscript.

We thank you again for considering our manuscript for publication in your journal.

Sincerely

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TITLE:

Plasmodium falciparum Gametocyte Culture and Mosquito Infection Through Artificial Membrane Feeding

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KEYWORDS:

malaria, anopheles, *Plasmodium falciparum*, erythrocyte, gametocyte, standard membrane feeding assay, SMFA, oocyst, sporozoite

SUMMARY:

Detailed investigations on mosquito stages of malaria parasites are critical to design effective transmission blocking strategies. This protocol demonstrates how to effectively culture infectious gametocytes and then feed these gametocytes to mosquitoes to generate mosquito stages of *P. falciparum*.

LONG ABSTRACT:

Malaria remains one of the most important public health problems, causing significant morbidity and mortality. Malaria is a mosquito borne disease transmitted through an infectious bite from the female *Anopheles* mosquito. Malaria control will eventually rely on a multitude of approaches, which includes ways to block transmission to, through and from mosquitoes. To study mosquito stages of malaria parasites in the lab, we have optimized a protocol to culture highly infectious *Plasmodium falciparum* gametocytes, a parasite stage required for transmission from the human host to the mosquito vector. *P. falciparum* gametocytes mature through five morphologically distinct steps, which takes approximately 1-2 weeks. Gametocyte culture described in this protocol is completed in 15 days and are infectious to mosquitoes from days 15-18. These protocols were developed to maintain a continuous cycle of infection competent gametocytes and to maintain uninterrupted supply of mosquito stages of the parasite. Here, we describe the methodology of gametocyte culture and how to infect mosquitoes with these parasites using glass membrane feeders.

INTRODUCTION:

Malaria is caused by *Plasmodium* parasites and is transmitted to their vertebrate hosts via infectious bite of female *Anopheles* mosquitoes. According to the 2019 World Health Organization (WHO) report, there were an estimated 405,000 deaths, from a total of 228 million cases of malaria[1]. Most of the malaria related deaths were concentrated in the African region, especially among children below five years of age. While the overall incidence rate of malaria has declined globally from 2010, in recent years decline has plateaued and additional control strategies are urgently needed to eliminate the disease.

Cyclic asexual blood stages of malaria parasites cause disease pathogenesis and a small subset of these differentiate into female and male gametocytes. *Plasmodium falciparum (P. falciparum)* gametocytes are unique in nature as they take 7-10 days to develop through five morphologically distinct stages. Immature gametocytes from stage I to IV are sequestered in bone marrow parenchyma and largely remain absent from peripheral circulation[2–5]. Erythrocytes infected with mature stage V gametocytes are released in the bloodstream and freely circulate to be taken up by mosquitoes. Once inside the mosquito midgut, gametocytes are activated, through a change in temperature and exposure to the midgut environment, transform into female and male gametes and begin development of the mosquito stages, which culminates with the infective stages of sporozoites in the mosquito salivary glands[6,7].

Since Trager and Jenson[8] described a standardized method to culture *P. falciparum*, studies on the asexual blood stages have greatly advanced. However, the lack of a reliable culture system for sexual stages has made it difficult to study *P. falciparum* gametocytes, transmission biology and mosquito stages. In recent years, several methods have been published which have aided laboratories in establishing gametocyte cultures[9–12]. This manuscript describes standardized and reliable protocol to culture *P. falciparum* gametocytes that can represent a valuable resource for the malaria research community. This method enables the robust production of mature and infectious gametocytes which along with a standardized mosquito feeding protocol, results in highly reliable mosquito infectivity. These methods were established to maintain uninterrupted supply of gametocytes, and mosquito stage parasites. In this manuscript, we describe a thorough gametocyte culture protocol (**Figure 1**), preparation of glass membrane feeders and infection of mosquitoes using these membrane feeders (**Figure 2**), dissection of midgut (**Figure 3**) and salivary gland of mosquitoes (**Figure 4**), and quantification of infection in mosquito after midgut and salivary gland dissection.

PROTOCOL:

Blood collections described below have been approved by the Institutional Review Board of Johns Hopkins University. *P. falciparum* is cultured in fresh RBCs under sterile conditions in a biosafety level 2 (BSL2) facility and caution is used to handle biological materials. After each step involving blood or blood products, every plasticware or glassware is rinsed with 10% bleach within the hood prior to proper disposal.

1. Reagents and preparation

1.1. Use parasite isolate *P. falciparum* NF54 (see **Table of Materials**) that can produce infectious gametocytes for up to two months while in continuous culture.

NOTE: Not all culture adapted parasite lines produce gametocytes, and low passage NF54 isolates are most consistent.

1.2. O^+ Erythrocytes: Dilute whole blood by adding equal volume of RPMI 1640 media and centrifuge at 500 *x g* for 5 min at room temperature in a swing out rotor with deacceleration set at 0. Carefully remove the supernatant and buffy coat (a white band between plasma and packed erythrocytes, which contains most of white blood cells and platelets) and add equal volume of RPMI. Repeat the washing step twice and after the final wash add one pellet volume of RPMI to get 50% hematocrit for storage at 4 °C.

NOTE: Gametocytes mature over a period of two weeks which makes it critical to begin cultures with fresh erythrocytes, typically erythrocytes drawn within a week works well.

1.3. O^+ Human Serum: Pool at-least 6 units of serum together to minimize the effect of normal variation in serum from different individuals. Sterilize pooled human serum using 0.2 µm filtration flasks. Aliquot in portions of 50 mL or less based on the need and store at -20 °C.

NOTE: Erythrocytes and serum needs to be from a compatible blood type for *P. falciparum* cultures.

1.4. 500x Hypoxanthine (100 mL) solution: Dissolve 0.5 g of hypoxanthine in 100 mL of 1 M NaOH. Filter sterilize and make 5-10 mL aliquots for storage. Stocks can be stored up to a year at -20 °C and up to 2 weeks at 4 °C.

1.5. Sodium bicarbonate (Optional): Dissolve 7.5 g sodium bicarbonate in 100 mL of deionized, tissue culture grade water and filter sterilize with 0.2 µm filter.

NOTE: This protocol uses the candle jar method to provide microaerophilic conditions for *P. falciparum* in vitro culture and does not require sodium bicarbonate. However, if parasites are cultured using a malaria gas mix (5% O_2, 5% CO_2 and 90% N_2), it is important to supplement culture media with 0.2% sodium bicarbonate.

1.6. Complete media: To prepare 500 mL of complete media, add 1 mL of hypoxanthine solution and 50 mL of pooled human serum to 500 mL of RPMI 1640. Add 15 mL of 7.5% sodium bicarbonate if using a malaria gas mixture. Store complete media at 4 °C and discard if the color of complete media changes from orange to pink. To avoid waste, make enough complete media to be used within three days.

1.7. Exflagellation media (Optional): Make exflagellation or ookinete media by dissolving 200 mg $NaHCO_3$, 5 mg Hypoxanthine and 100 µL of xanthurenic acid (from 100 mM stock in water) to

100 mL of incomplete media (RPMI 1640 with glutamine and HEPES).

NOTE: Exflagellation is the process of male gamete formation inside the midgut of female Anopheles mosquito few minutes after it takes blood meal infected with gametocytes. Male gametocytes of Plasmodium give rise to 8 male gametes after an exflagellation event. In vitro, this process occurs spontaneously when culture temperature is lowered to room temperature (RT) and can be observed in cultured mature gametocytes.

1.8. N-Acetylglucosamine (Optional): Make 500 mM stock solution of N-acetylglucosamine in water or incomplete media, aliquot and store at -20 °C.

1.9. NaCl solutions: Dissolve 0.9 g, 1.6 g and 12 g NaCl in 100 mL of tissue culture grade deionized water to make 0.9%, 1.6% and 12% NaCl solutions. Filter sterilize with 0.22 µm filter and store at 4 °C.

2. *P. falciparum* asexual stage culture

2.1. To begin parasite culture, remove a low passage frozen vial of *P. falciparum* NF54, from the liquid nitrogen tank and quickly thaw in water bath set at 37 °C.

2.2. Transfer contents (~1 mL) to a 50 mL sterile centrifuge tube and dropwise add 0.2 volume of prewarmed 12% NaCl, while gently shaking the tube to ensure even mixing. Incubate for 5 min at RT with intermittent gentle shaking.

2.3. Add 9 volumes of 1.6% NaCl dropwise, while continuing gentle mixing. Centrifuge the contents at 500 *x g* for 5 min at RT, carefully discard the supernatant. Add 9 volumes of 0.9% NaCl dropwise, while making sure to consistently mix the parasite pellet. Centrifuge again at 500 *x g* for 5 min at RT. Remove the supernatant and resuspend parasites into 5 mL of complete media.

2.4. Transfer contents to one well of a 6 well tissue culture plate and add 100-200 µL of packed RBCs.

2.5. Incubate parasite culture at 37 °C in a candle jar or in a modular incubator chamber purged with special gas mix of 5% O_2, 5% CO_2 and 90% N_2.

2.6. Replace media every day and monitor the growth by making a thin blood smear by drawing a few microliters from settled RBC layer after the culture supernatant has been aspirated during regular media change.

2.7. Use sterile glass Pasteur pipette to draw and then tap in the center of the glass slide to transfer a drop of culture to the slide. Place another glass slide in front of the of the drop and draw it back to contact the RBCs, quickly pushing it forward in one motion to make thin smear of RBC monolayer.

2.8. Place the slide horizontally on a drying rack and let it air dry. Fix the blood smear by dropping absolute methanol onto the smear. Allow the fixed smear to dry completely and then carefully pour 10% Geimsa-stain freshly diluted in water, until blood smear is completely covered. Allow cells to stain for approximately 15 min.

2.9. Wash off the excess stain by rinsing the slide under clean tap water and allow the slides to dry in vertical position.

2.10. Determine parasitemia by viewing thin blood smear on a microscope using oil immersion 100x objective. Count the number of infected erythrocytes among a total of at-least 500 RBCs to determine percent parasitemia.

3. *P. falciparum* gametocyte culture

NOTE: Gametocyte cultures take two weeks to produce mature gametocytes infectious to mosquitoes. The steps of the gametocyte culture are outlined in **Figure 1**. *P. falciparum* isolates usually lose the ability to produce gametocyte after long term in vitro culture[13]. To ensure quality of gametocytes, culture should be initiated from low passage feeder culture, not more than 2 months old since thawing. Pre-warm media to 37 °C and perform all the procedures on a slide warmer set at 38 °C. Make sure gametocyte cultures are not out of incubator for extended period to minimize temperature fluctuations.

3.1. Seed the gametocyte culture (day 0) using mixed asexual stage feeder culture at 0.3-1% parasitemia at 4% hematocrit.

3.2. To set up a six well gametocyte culture, centrifuge 5 mL of feeder culture at 500 *x g* for 5 min at RT. Discard the supernatant and resuspend the pellet in 30 mL of complete media.

3.3. Add 1.2 mL of packed RBCs, mix, and dispense 5 mL to each well of the 6 well plate and incubate in a candle jar at 37 °C.

NOTE: Gametocyte culture set up described above is based on a mixed asexual stage feeder culture at 5% parasitemia.

3.4. Change media daily for 15-18 days, without the addition of fresh blood, by carefully aspirating about 70-80% culture supernatant to avoid removing blood cells.

3.5. Add 5 mL of fresh complete media to each well. While changing media, slowly add media using a serological pipette against the wall of the well to avoid disturbing the settled RBC layer.

NOTE: Because 1-2 mL of the culture medium will be left during media change, total volume of culture will be between 6-7 mL, after day 1 of gametocyte culture. While adapting this protocol, it is advisable to make blood smears every alternate day to make sure parasites are healthy. Making a blood smear can often deplete the number of cells in culture. To avoid this, draw a very

small volume.

3.6. To quantify mature gametocytemia, make blood smear between days 15-18 and count the number of mature gametocytes among total number of cells.

NOTE: Mature gametocytes can easily be identified with their classic crescent shape with smooth rounded ends (**Figure 5B**).

3.7. Count a minimum of 1,000 RBCs and calculate the percentage of RBCs infected with mature gametocytes.

3.8. To quantify exflagellation events, take 200 µL of gametocyte culture and centrifuge at 500 *x g* for 5 min at RT in a pre-warmed tube. Resuspend the pellet in 20 µL of exflagellation media and transfer to a glass slide with cover slip.

3.9. After 15 min incubation at room temperature, begin counting exflagellation centers in phase contrast mode using 10x objective. Count exflagellation centers in at-least four fields to calculate exflagellation events.

NOTE: A detailed methodology of exflagellation assay has been described earlier by Delves et al[10]. More than 20 exflagellation events per field is considered suitable for membrane feeding assay.

3.10. Ensure that gametocyte cultures have low levels of residual asexual stages. If the experiment requires pure gametocytes, treat the culture with 50 mM N-acetylglucosamine.

3.11. Add 0.5 mL of N-acetylglucosamine from 10x stock solution per well (5 mL) for a minimum of 3 days while changing media to clear residual asexual stages.

NOTE: N-acetylglucosamine can block invasion of sexually committed merozoites too, treatment should be initiated only after day 7 of gametocyte culture.

4. Mosquito infection using standard membrane feeding assay (SMFA)

NOTE: Gametocytes grown in vitro can be fed to mosquitos using glass membrane feeders. Setting up of the blood feeding apparatus is shown in **Figure 2**. As described above, always maintain gametocytes at 37 °C to avoid activation before they are ingested by mosquitoes. Prewarm plasticware, reagents and equipment's used with gametocyte culture to 37 °C.

4.1. Starve 3-7-day old female *Anopheles* mosquitoes by removing their sugar water for 6.5 h or overnight before membrane feeding.

4.2. Transfer them using motorized or mouth operated aspirators to paper cups covered with double layer of fine mesh fabric. Alternatively, knock down mosquitoes in a cold room or

refrigerator to transfer them to the paper cups.

NOTE: One-pint size cup can be used to feed up to 100 mosquitoes.

4.3. Centrifuge washed blood at 500 *x g* for 5 min at RT and discard supernatant. Add an equal volume of freshly thawed human serum to reconstitute the whole blood. Transfer the blood and serum mix to the water bath set at 37 °C for 30 min.

4.4. Transfer gametocyte culture to pre-warmed 15 mL plastic tube and centrifuge at 600 *x g* for 5 min at 37 °C. Carefully aspirate the culture supernatant and make thin blood smear to determine mature gametocytemia as described above.

4.5. To make the final blood feed, dilute gametocyte pellet to a desired concentration using reconstituted whole blood. Keep the blood feed at 37 °C until mosquitoes and glass feeders are ready.

NOTE: Mature gametocyte concentrations between 0.02 to 0.3% will provide consistent mosquito infectivity. However, it is advisable to optimize gametocytemia by feeding various concentrations to different cups of mosquitoes.

4.6. Ensure that the glass membrane feeders have two openings, a narrow top opening to pipette the infected blood and a bottom opening for membrane attachment. Cut a paraffin film into squares, stretch to an even thickness and stick on to the opening of feeder to create sealed compartment for the blood feed.

4.7. Attach membrane feeders to a circulatory water-bath by connecting tubing on each side to allow for the passage of warm water through the jacket around membrane feeders.

NOTE: Several glass feeders can be connected in series to accommodate for multiple feeding conditions.

4.8. After all the feeders are connected to the water bath, turn it on and inspect for any leaks.

4.9. Place feeders in the center of netting on mosquito cups with membrane-side down and secure feeders using clamps or tape. Make sure membrane feeders are not tilted to any side to allow even distribution of the blood feed and close contact of the entire feeder with the netting on cups, to allow mosquitoes access from inside the cup.

4.10. Pipette about 200–1000 µL of blood feed into membrane feeders.

NOTE: Volume of the blood feed will vary based on the number of mosquitoes and size of the membrane feeder. For 14 mm glass feeder and 50 mosquitoes use 200 µL of blood feed.

4.11. Make sure loading was done properly and blood feed was overlaid on the paraffin film.

4.12. Allow mosquitoes to feed for about 30 min with intermittent monitoring.

NOTE: Gently blow mosquitoes with mouth to provide CO_2 which will induce improved blood feeding.

4.13. Remove unfed mosquitoes by knocking them down in a cold room. Visibly inspect mosquitoes for bulge and redness in the abdomen as a sign of fresh blood meal. Alternatively, use a mouth operated aspirator to selectively remove unfed mosquitoes.

4.14. Discard unfed mosquitoes, after soaking them in 70% ethanol and put fed mosquitoes back into the mosquito cup.

4.15. Double cage mosquito cups and transfer them to high containment incubator specified for mosquitoes infected with human malaria parasite *P. falciparum*.

4.16. Place cotton pads soaked in 10% sucrose on the mosquito cups to provide them with sugar meal. Replace cotton pads every other day, until mosquitoes are dissected.

5. Mosquito mid-gut dissection and oocyst load quantification

NOTE: A schematic of midgut dissection is shown in **Figure 3**.

5.1. 7-8 days post blood feeding, transfer mosquito cups to 4 °C for 10 min to knock down mosquitoes.

5.2. Transfer mosquitoes to be dissected to a Petri dish on ice using fine-tipped forceps.

5.3. Soak mosquitoes in 70% ethanol for 1-2 min to euthanize them. Add PBS to the Petri dish to wash off the ethanol after all the mosquitoes are euthanized.

NOTE: Before adding PBS make sure all the mosquitoes are dead.

5.4. Mount a glass slide on dissecting microscope and pipette 100 µL of PBS in the center. Carefully transfer one mosquito using forceps into the PBS and leave the rest of the mosquitoes in the Petri dish on ice.

5.5. Using fine-tipped forceps, hold the third segment of the abdomen from the posterior end of the mosquito and with second forceps hold the junction between the thorax and abdomen. Gently pull the abdomen until the midgut is fully exposed.

5.6. Discard the rest of the mosquito tissue and transfer the midgut to a clean slide containing a few drops of PBS.

5.7. When the desired mosquitoes have been dissected, carefully remove the PBS using a pipette and stain the midgut with 0.2% mercurochrome for 2-5 min.

5.8. Remove excess mercurochrome and line-up midguts on the slide so that they can be easily visualized under the light microscope.

5.9. Place a cover slip and count oocysts on each midgut under 10x objective. Oocysts stain pink and are circular in shape (**Figure 6C**).

6. Mosquito salivary gland dissection and sporozoite load quantification

NOTE: A schematic of salivary gland dissection is shown in **Figure 4**.

6.1. 14-18 days post blood feeding, place mosquito cups at 4 °C for 10 min.

NOTE: It is important to wait till mosquitoes stop moving.

6.2. While waiting for mosquitoes to stop moving, prepare tools for dissection.

6.3. Place a glass plate on a dissection microscope stage. Prepare 2 sets of 25 G needles on 1 mL syringes and a 9" Pasteur pipette and rubber bulb. Place 70% EtOH and dissection medium (HBSS, L-15 or PBS) in a 6 well plate.

6.4. In the cold room, transfer anesthetized mosquitoes in a 6 well plate containing 70% EtOH. Gently move mosquitoes with forceps to make sure all mosquitoes are soaked, then transfer mosquitoes to the dissection medium by using forceps to wash off 70% EtOH.

6.5. Move to the dissection room and place mosquitoes on a dissection microscope stage.

6.6. Remove excess medium from mosquitoes, add new medium as necessary during dissection. Avoid mosquitoes from drying out yet too much liquid makes dissection more difficult.

6.7. Using 2 syringes with 25 G needles, hold mosquito thorax and head, and gently pull the head upward to pull salivary glands from thorax.

6.8. Disconnect salivary glands from head and thorax with a needle.

6.9. Temporary place salivary glands in a separate droplet of medium on the glass plate.

6.10. After dissecting 15-20 salivary glands, collect them in a low retention tube using the Pasteur pipette.

NOTE: Once salivary glands enter the wide part of the Pasteur pipette, they will stick to the glass and it is difficult to get them out.

6.11. Pellet salivary glands by short pulse spin in a table-top centrifuge. Remove the dissection medium without disrupting the pellet and Add 100 µL of new dissection medium.

6.12. Grind salivary glands with a small homogenizer for 1 min to obtain sporozoites.

6.13. Place 10 µL of dissection medium containing sporozoites on a hemocytometer.

NOTE: Depending on the number of salivary glands, one may need to dilute the sporozoite solution to 1:10 to 1:50 with medium before counting.

6.14. Count the number of sporozoites in two of the four quadrants and calculate number of sporozoites/mosquito:

$$\frac{(1st\ quadrant + 2nd\ quadrant)}{2} \times 10^{4} = Number\ of\ sporozoites/ml\ (Sporozoites/ml)$$

$$(Sporozoites/ml) \times volume\ of\ medium \times dilution = Total\ number\ of\ sporozoites$$

$$\frac{Total\ number\ of\ sporozoites}{number\ of\ mosquitoes\ dissected} = Average\ sporozoite\ number/mosquito$$

REPRESENTATIVE RESULTS:

Here we present results from a series of membrane feeds using *P. falciparum* NF54 gametocyte cultures generated using the protocol above (see (**Figure 5**). Gametocyte culture was initiated with approximately 0.5% mixed stage asexual culture on Day 0, which grew to a peak parasitemia of approximately 15% by Day 4 and Day 5. As shown in **Figure 5A** at this high parasitemia, the parasites are stressed and the asexual stage culture crashes. However, this stress concomitantly results in the induction of gametocytogenesis. Early gametocytes appeared after Day 6 and Day 7 and asexual parasitemia slowly declined but remained at a low level. Presence of asexual stage parasites did not affect mosquito feeding experiments. However, if gametocytes are to be used in experiments which require pure cultures, such as drug sensitivity assays and proteomic or transcriptomic studies, residual asexual stages can be removed by treatment with 50 mM N-acetylglucosamine. The majority of gametocytes mature to stage V by Day 15 at which time they become infectious to mosquitoes and were ready to be fed. Representative images of Giemsa-stained blood smears at different time points after initiation of gametocyte culture are shown in **Figure 5B.**

Between 8 to 10 days after blood feeding, mosquitoes were dissected to determine the prevalence and intensity of infection. Prevalence is the percentage of fed mosquitoes that have oocysts while intensity is the number of oocysts found in each mosquito. Both are important indicators of the success of the feed. Data from 5 independent mosquito feeds are shown in

Figure 6. Feeds were chosen to show the normal variation in the levels of infectivity after feeding with 0.3% mature gametocytes from day 16 culture of *P. falciparum* NF54. Oocyst intensities provides quantitative data to determine mosquito infectivity of gametocytes and prevalence shows the percentage of fed mosquitoes that became infected. These data can be used to evaluate transmission blocking agents and to identify and characterize targets for transmission blocking vaccines and drugs. Number of oocysts varied both within and between experiments and required 25-50 mosquitoes per cohort to determine the effect of various experimental conditions.

Oocyst intensities are considered as end point of most transmission blocking assays and strategies, however number of sporozoites are usually important for sporozoite biology and for liver stage studies. **Table 1** shows the average number sporozoites obtained per mosquito from 12 independent blood feeds. As shown in the table, average number of sporozoites were consistent, however, there was one experiment where we obtained zero sporozoites, representing occasional failure of the assay.

FIGURE LEGENDS

Figure 1: Workflow of *P. falciparum* gametocyte culture and membrane feeding protocol.

Figure 2: Mosquito blood feeding set-up. (**A**) Glass feeder and rectangular piece of paraffin film (**B)** Two glass feeders displayed before and after parafilm membrane attachment (**C**) Glass feeder on top of mosquito cup and connected with circulation water bath. (**D**,**E**) Pipetting of blood feed into the glass feeder (**F**) Bottom view of glass feeder showing homogenous distribution of blood feed (**G**) Several mosquitoes feeding through parafilm membrane. (**H**) Top view of mosquito cups after feeding, showing drops of blood es excreted by the feeding mosquitoes at the bottom of cup. Scale bar = 10 mm.

Figure 3: Graphical representation showing steps of mosquito midgut dissection.

Figure 4: Graphical representation showing steps of mosquito salivary gland dissection.

Figure 5: *P. falciparum* gametocyte culture and oocyst visualization on infected mosquito midgut. (**A**) Time course of 15 day gametocyte culture, showing steep multiplication of asexual stages to peak parasitemia within first 4 days, followed by gametocytogenesis and maturation over time. (**B**) Geimsa-stained thin blood smear showing various stages of gametocyte culture, day 1 early asexual stage, day 4 peak asexual parasitemia, day 6 stressed culture due to high parasite load, days 9 & 12 early gametocytes and day 15 showing mature male and female gametocytes. Morphologically early stage gametocytes were indistinguishable from asexual stages, but late stage II showed the crescent shape with pointed ends and elongated as parasite developed into stage III and IV. Mature stage V gametocytes, however, was characterized by classic crescent shape with rounded ends and minimal host cell visibility[14]. Scale bar = 10 μm

Figure 6: *P. falciparum* oocysts counts per mosquito midgut: (**A**) Graph shows oocyst counts

over the years, for each experiment, mosquitoes were fed with 0.3% gametocytes and midguts were dissected on day 8 post blood feed. Each dot represents the number of oocysts from individual midguts and the horizontal line represent median value. (**B**) Table shows mean oocyst count, prevalence of infected mosquitoes and range of infection. (**C**) Pictures showing oocysts on mosquito midgut from two separate blood feeding experiments at different magnifications. Scale bar = 150 μm.

Table 1: Average number of salivary glands sporozoites per mosquito for 12 independent cycles over a 2-year period. *A. stephensi* mosquitoes were fed with 0.3% *P. falciparum* gametocytes and 15-20 mosquitoes were dissected between days 14-17 post blood feeding. Average sporozoite counts per mosquito are shown.

DISCUSSION:

Methods described here have been successfully used at the Johns Hopkins Malaria Research Institute for more than 10 years[15–22]. Gametocytes produced using this protocol have been used for high throughput gametocytocidal assays[22], for proteomic[15], as well as for transcriptomic[23] studies. However, a major reason to develop these methods is to use the mature gametocytes to infect mosquitoes for studies on mosquito stages and sporozoites[23–26]. This manuscript describes a detailed protocol of generating mature *P. falciparum* gametocytes and infection of mosquitoes using glass membrane feeders. These methods are critical for any laboratory working on transmission blocking strategies, sporozoite stages and pre-erythrocytic liver stages of *P. falciparum.*

Ifediba and Vanderberg in 1981 described long term culture of *P. falciparum*, in the presence of 50 μg/mL hypoxanthine which produced highly infectious mature gametocytes[27]. Since then there have been numerous publications describing methods to produce gametocytes for different applications[9–12]. Most of these publications utilize previously described gametocytogenesis-inducing conditions to increase yields. Using conditioned media, stressing the culture by sudden parasitemia increase, drop in hematocrit to mimic anemia, red blood cell lysis and log phase repression by bulk up, can be used to induce gametocytogenesis. The method described here is simple and time tested. Initiate gametocyte culture with 0.3-1% asexual stage parasitemia at 4% hematocrit and change media every day until day 15-18. To achieve consistent results, it is critical to begin gametocyte culture with low passage asexual stage parasites, use fresh RBCs (<1 week) and making sure that the culture temperature does not fluctuate during media change. Since *falciparum* gametocyte development process occurs sequestered in static conditions of extravascular spaces in bone marrow[4,5], it's important to not disturb settled RBC layers throughout the culture period.

Culturing *P. falciparum* gametocytes with consistency is demanding but getting them to infect mosquitoes presents another level of complexity. Membrane feeding is subject to several variables other than gametocytes, such as age and fitness of mosquito, midgut microbiota, and feeding behavior[28,29]. Usually SMFA data show a high degree of variability and require large number of mosquitoes to identify effects of different experimental conditions[11,28]. Using low passage culture for gametocytes, healthy 3 – 6-day old mosquitoes and optimized membrane

feeding protocol can help with variations in oocyst counts.

The protocol described here for both gametocyte cultivation and membrane feeding, has been optimized over many years. These methods provide a detailed description for obtaining mature transmission competent gametocytes, standard membrane feeding assay, mosquito midgut dissection and oocyst quantification as well as salivary gland dissection and sporozoite quantification. These protocols are consistent in terms of the number of gametocytes needed to provide reliable mosquito infectivity and robust oocyst counts and sporozoite yields.

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DISCLOSURES:

The Authors have nothing to disclose

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598 repression during the maturation of malaria parasite sporozoites. *Nature Communications*. **10**,
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615
616

Figure 1

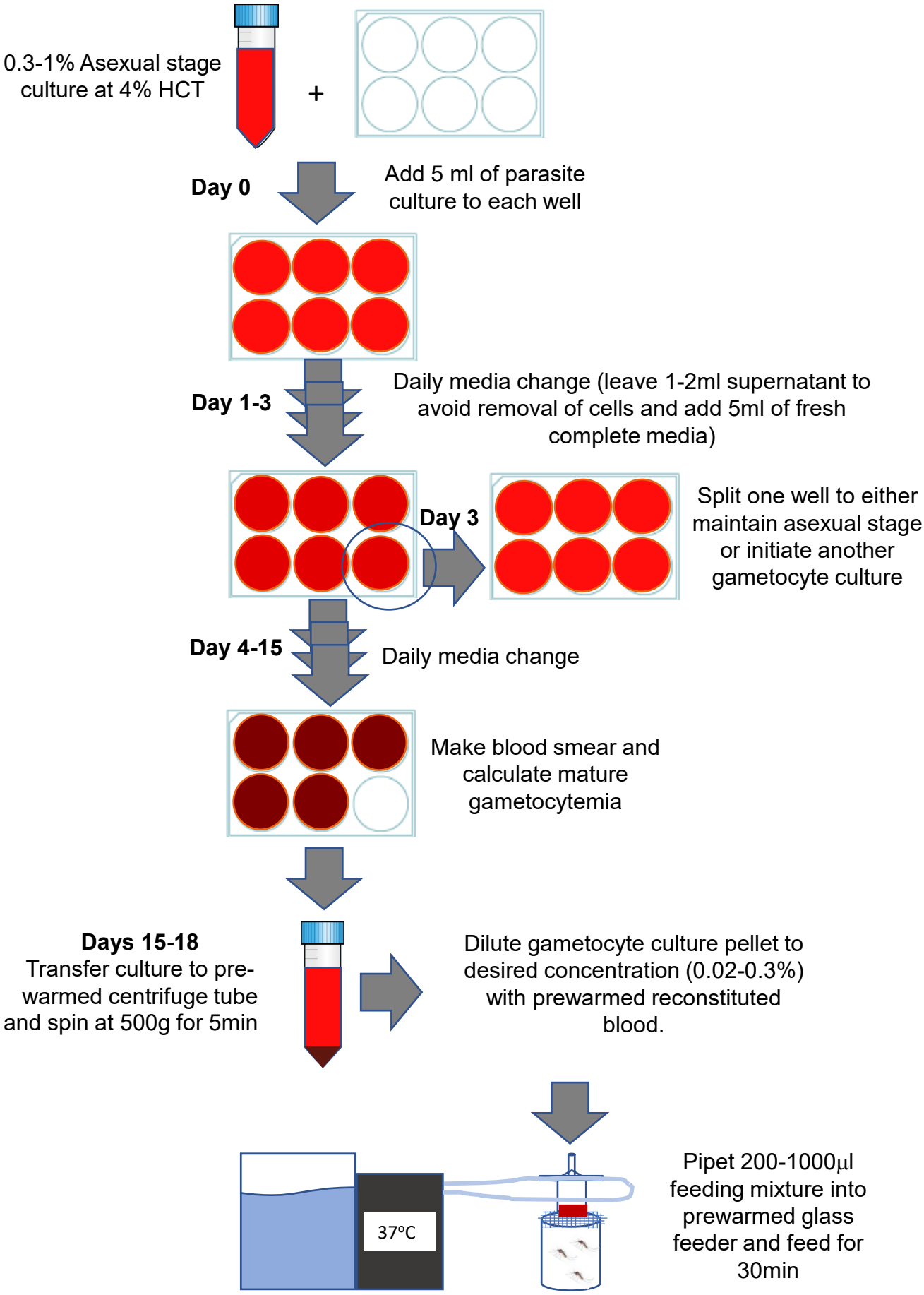


Figure 2

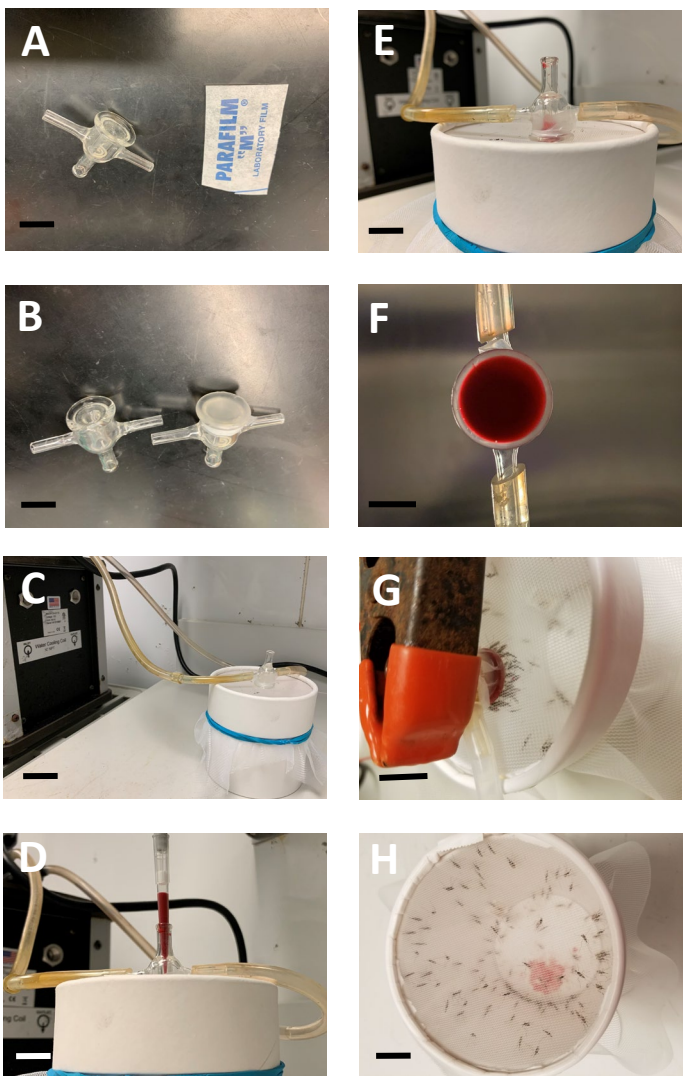


Figure 3

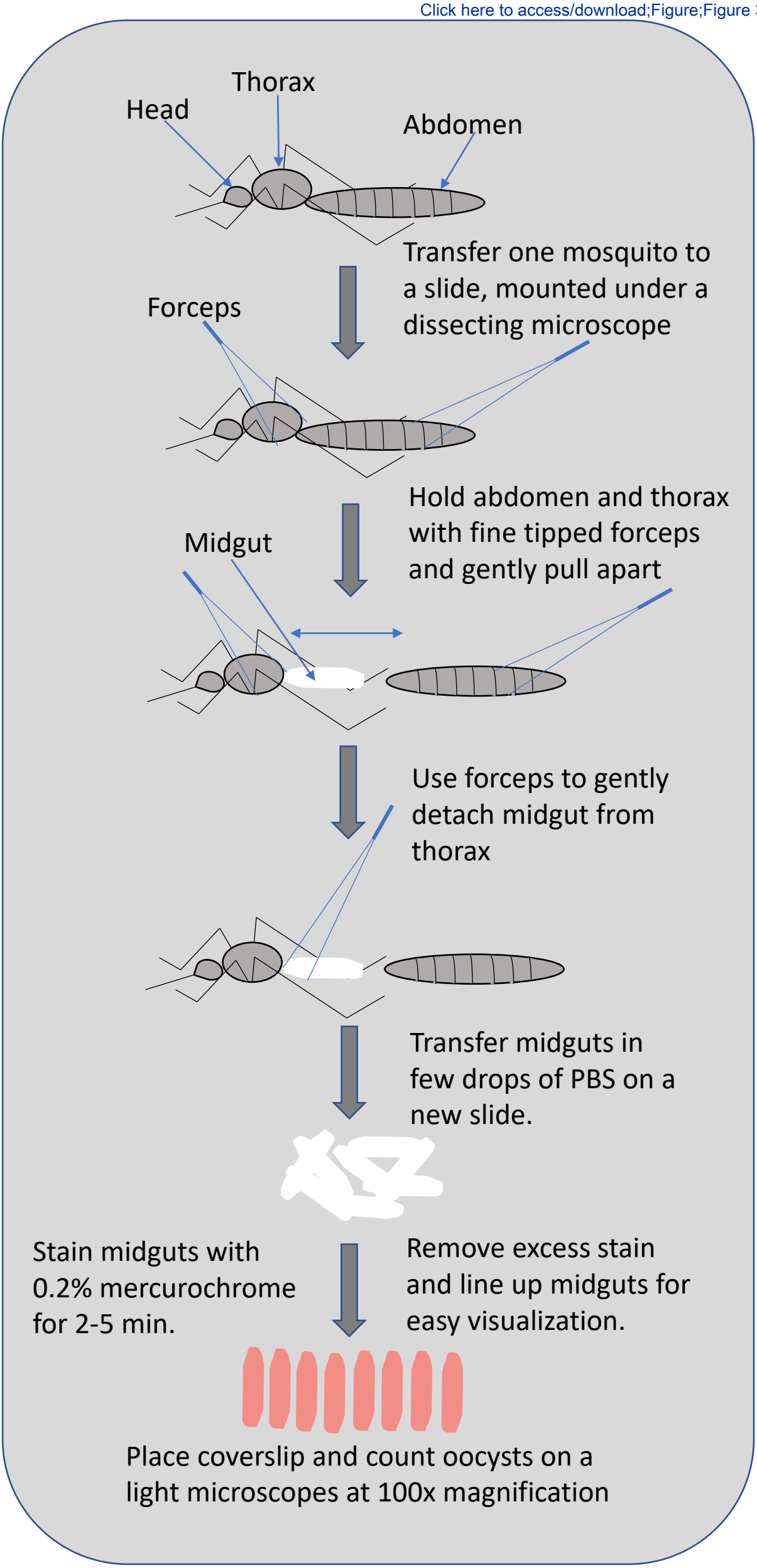
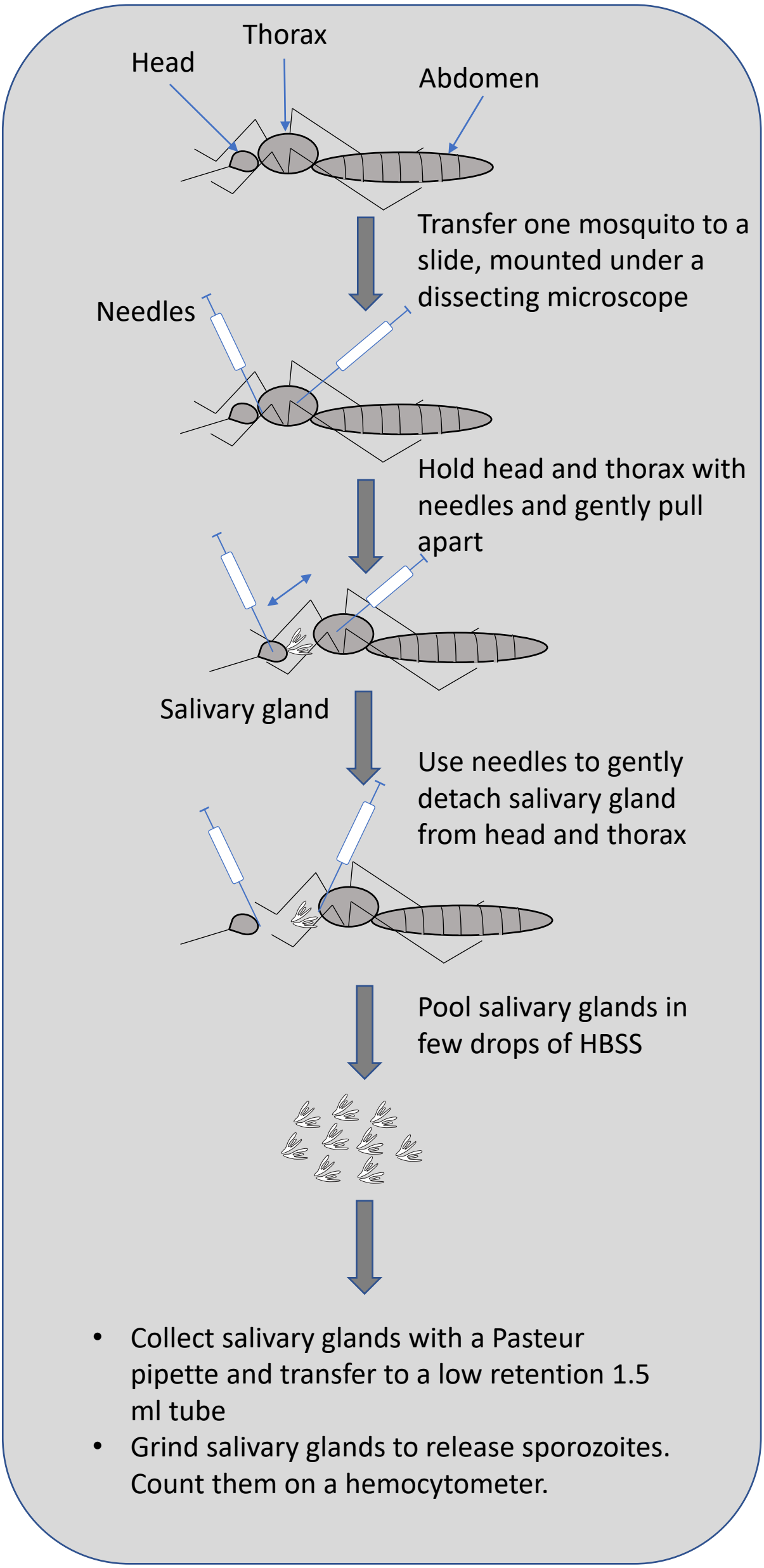
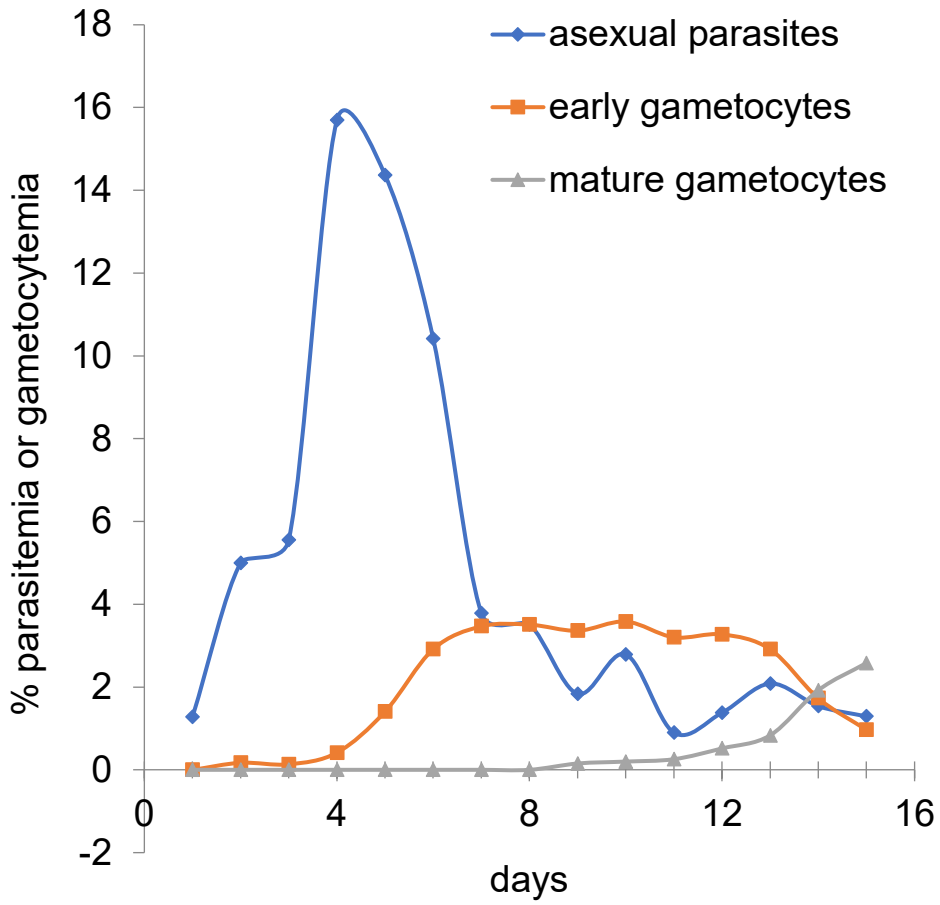


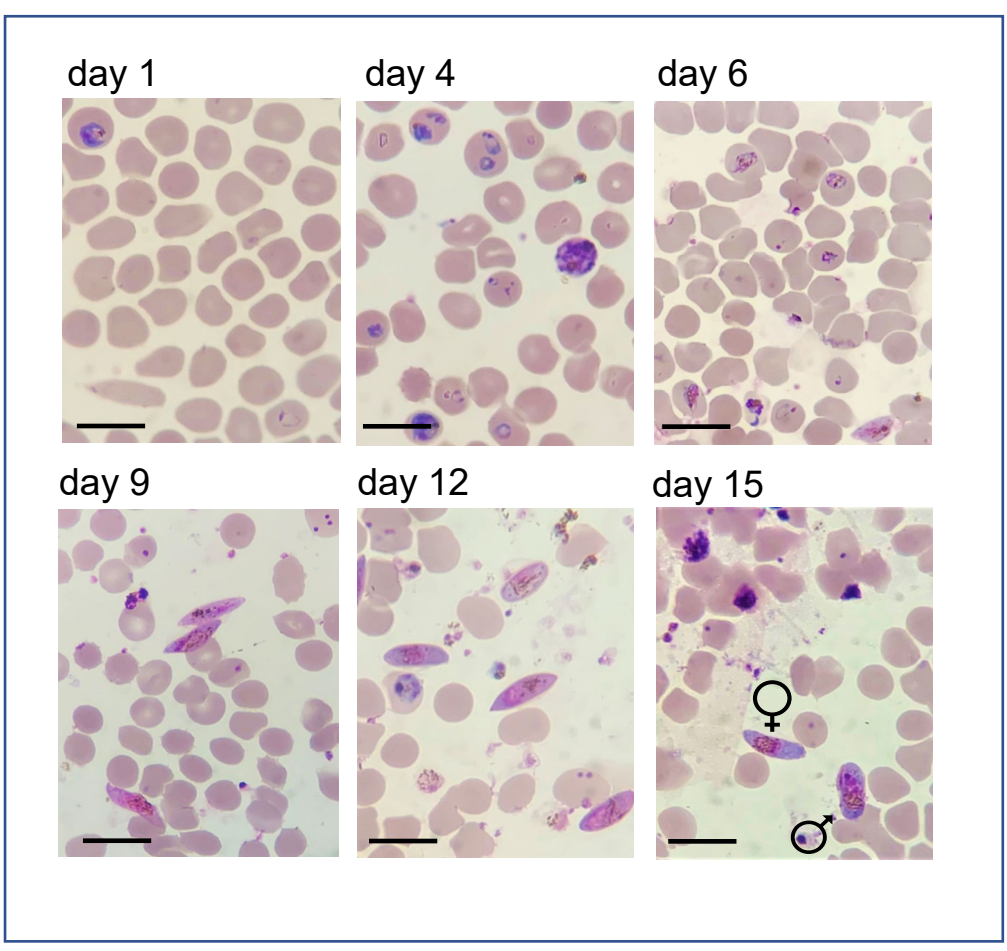
Figure 4



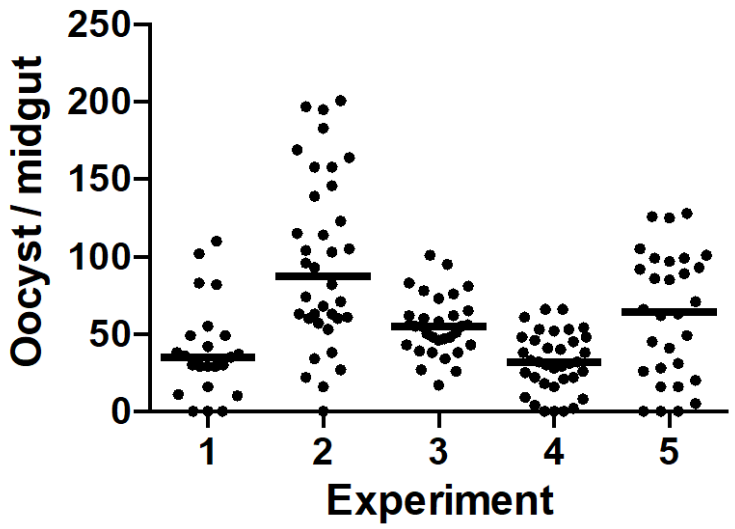
A



B



A



B

Experiment	Mean oocyst number	Prevalance (range)
1	24	88 (0-110)
2	36	92 (0-201)
3	32	100 (17-101)
4	36	89 (0-66)
5	30	90 (0-128)

C

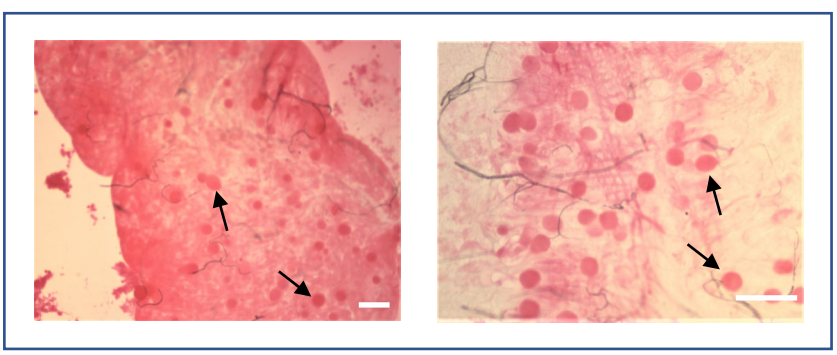


Table 1. Average number of salivary glands sporozoites per mosquito for 12 independent cycles over a 2-year period. *A. stephensi* mosquitoes were fed with 0.3% *P. falciparum* gametocytes and 15-20 mosquitoes were dissected between days 14-17 post blood feeding and average sporozoite counts per mosquito are shown.

Experiment	Day of dissection (post blood feed)	Mean sporozoite/mosquito
1	14	42, 000
2	14	40, 000
3	14	42, 750
4	15	25, 411
5	15	33, 750
6	14	15, 750
7	16	0
8	15	33, 200
9	14	56, 333
10	14	45, 333
11	17	43, 750
12	16	56, 000

Table of Materials

Click here to access/download;Table of Materials;Materials list.xlsx

Name	Company	Catalogue
10% Sugar solution		
10ml serological pipet	Falcon	357551
15 ml conical tube	Falcon	352096
1ml serological pipet	Falcon	357521
25 ml serological pipet	Falcon	357535
37°C Incubator		
50 ml conical tube	Falcon	352070
5ml serological pipet	Falcon	357543
6 well tissue culture plates	Falcon	353046
70% Ethanol		
9" glass pipet	Fisherbrand	13-678-6B
Anopheles Mosquitoes	JHMRI, Insectary core	
cell counter		
Circulating water bath		
fine tip forceps	Fisherbrand	12-000-122
Geimsa stain	Sigma	GS1L
Glass desiccator		
Glass membrane feeder	Chemglass Life Sciences	CG183570
Glass slides	Fisherbrand	12-552-3
HBSS	Sigma	H6648
Human Blood O+	JHU	
Human Serum O+	Interstate blood bank	
Hypoxanthine	Sigma	H9337
Mercurochrome	Sigma	M7011
Micro Pipette		
Microscope	Olympus	
Mosquito cups	Neptune cups	
N-acetylglucosamine	Sigma	A3286
Netting		
Parafilm		
PBS		
Petri dish	Thermo Scientific	249964
Pipet tips		
Pipetman		
Plasmodium falciparum NF54	BEI Resources	MRA-1000
RPMI 1640	Corning	CV-041-CV
Slide warmer		
Sodium bicarbonate	Sigma	S6297
water bath		
Xanthurenic Acid	Sigma	D120804

Comments
We use A. stephensi or A. gambiae (keele)
Wash RBCs three times with RPMI and refrigerate at 50% heamatocrit
Pool at-least 6 units of serum from different donors and freeze down aliquots at -20°C.
Make 500x stock in 1M NaOH
Prepare 1% stock solution in PBS that can be diluted to 0.1% when needed
Any microscope with 10x, 40x and 100x objective will work.
Optional and needed only when pure gametocytes are required.
Make sure it can contain mosquitoes and allow blood feeding
Freeze down large numbers of early passage culture to make sure you have a constant supply
Media contains glutamine and HEPES
Optional for media, add only when using malaria gas mix during culture incubation
For flagellation media

We would like to thank the editorial team and all the reviewers for their comments and suggestions, we have addressed most of the comments. We feel most of the comments and inputs were very useful and have helped improve the manuscript. Below we have included point by point response to each comment from editorial team and reviewers.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have proofread the manuscript for typographical and grammar issues and believe language of the manuscript is much improved now.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

Manuscript is formatted accordingly

3. Please provide an email address for each author.

Email addresses of all the authors are provided

4. Please ensure that the Abstract is between 150-300 words and clearly states the goal of the protocol.

Abstract is edited as suggested

5. Please expand the Introduction to include all of the following with citations:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application

Introduction is edited as suggested

6. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).
All instances of personal pronouns are replaced

7. We cannot have non- numbered heading, subheadings, steps in the protocol section. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.

Numberings are reformatted as suggested

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

All of the steps are edited to be in imperative tense and NOTES are included for sentences that cannot be expressed in imperative tense.

9. Please write the steps as if you are describing someone how to do you experiment with all possible details in complete sentences.

Edited as suggested

10. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

Edited as suggested

11. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step.

Edited as suggested

12. For all centrifugation steps please include temperature and time as well

We have included temperature and time for all the centrifugation steps

13. Please ensure you answer the "how" question, i.e., how is the step performed?
14. 1.2: How do you wash the whole blood? How do you remove plasma and WBC? How do you ensure 50% hematocrit is present?
15. 2.1 How do you identify feeder asexual stage visually? How do you perform Giemsa staining?
16. 2..4: How do you make blood smear?

Details of the blood washing, microscopic visualization of parasite and Geimsa staining is included as suggested

17. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Part of protocol which should be on the video is highlighted

18. In the result section, please include the success rate of the technique. Please ensure that the result section is written as if you performed your experiment and now describing your results to show the effectiveness of your technique.

Results section is edited as suggested

19. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

We have not used any published figure, however citations of several publication using this protocol is included in results section.

20. As we are a methods journal, please ensure the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Discussion is formatted to include suggested content

21. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al.

References are added in JOVE format

22. For images with microscopes, please include a scale bar and define it in the figure legend.

Scale bar is added with images and defined in figure legend

23. Please sort the materials table in alphabetical order.

Materials table is sorted alphbetically

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This manuscript summarizes the process for Plasmodium falciparum gametocyte culture and transmission to mosquitos with sufficient detail to be replicated by someone familiar with malaria culture. The manuscript outlines detailed in vitro culture of the blood stages through verification of transmission in mosquitos. As this is from one of the world's leading malaria centers it is of great interest and relevant to those in the malaria community or those entering and therefore is of high value.

We are thankful for your words of encouragement.

Major Concerns:
I generally only have minor concerns with the written portion of the manuscript. As someone familiar with the process I found it easy to follow but a less experienced reader may want more detail concerning culture conditions including shaking vs. stationary culture or references to alternative methods and why these have been chosen.

We thank reviewer for suggestions, we have added more details in the protocol

Minor Concerns:
The majority of my concerns lie with simple grammatical mistakes detailed below. Otherwise, the the manuscript will be suitable for publication with accompanying video in my opinion.

Line 134: Change to "blood smears"
Line 135: Change to "a blood smear"
Line 138: same
Line 139: to avoid "this"
Line 142: count "the" number
Line 145: pictures of mature gametocytes?
Line 163: change to "mosquitos"

Line 164: add "," after "As described above"
Line 181: remove first comma
Line 237: should be "100x" no?
Line 242: Should be "The methods described here have been used in our laboratory for a long time.." although specific time length (i.e. for <10 years) would be best.
Line 282: Add "The" at beginning of sentence
Line 287: should be "processes"
Line 288: remove second comma
Line 292: variability should be "variables"
Line 298: Should be "The protocol"

Thank you for critical review of the manuscript and pointing out grammatical and typographical errors. Manuscript has been edited to correct all the linguistic errors.

Reviewer #2:
Manuscript Summary:
The manuscript entitled 'Plasmodium falciparum gametocyte culture and mosquito infection through artificial membrane feeding' by Tripathi et al, describes a detailed and seemingly simpler method of generating mature gametocytes and the subsequent membrane feeding needed to establish mosquito stages of P. falciparum. Because the authors have optimized and tested the protocol in their lab for over a long time their methodology could be a highly useful resource for labs working on the pre-erythrocytic stages of P. falciparum or developing transmission-blocking strategies.
Overall, the manuscript is well written, and the experimental design is presented in detail although some points could be better explained.

We are thankful to the reviewer for kind words

Major Concerns:
There should be figure (new figure or panel, reorder if necessary) displaying the feeder set-up

A new figure is added showing pictures of stepwise feeder set up

Figure 2C shows oocyst production on day 7 post-infection. How does this look at day 14-21? Also, please provide a description of how oocysts development is evaluated (e.g., size in µm, number, etc.). Also, indicate a description of the magnification.

Oocysts can be visualized between days 7 to 10 and after day 10 most of the oocyst will rupture and sporozoites will be released in hemolymph. Scale bar is added in the image to provide size reference.

The "maturation" of the sporozoites is not demonstrated in this work. A measurable way of quantifying the number of oocysts reaching maturation in the mosquito should be added. Do oocysts remain in the midgut on salivary gland extraction day? Mention ratio between the two glands (measure of how many have not matured yet).

A table showing average sporozoites per mosquito obtained with several independent experiments is added in the manuscript. We have not measured ration between both glands and unfortunately due to prevailing condition we cannot perform these detailed experiments. Purpose of this manuscript is providing a detailed yet simple protocol of obtaining mature gametocytes and generate robust mosquito stage infection.

Minor Concerns:
On Line 35, it should be World Health Organization (caps)

Line 38 and 39: The authors say there is a decrease in incidence since 2010, but there have actually been increases in total cases within the last few years. They might cite the WHO world malaria report.

Edited as suggested

In the reagents section (Page 1, Line 68), indicate how long reagents last at -20C and at 4C.

Suggested details are included

On Page 1, Line 79, what are WBC? Include abbreviation list.

More details are added to make it clear

The authors should include the average oocyst count per mosquito midgut and average sporozoite yield per mosquito. This could be helpful for the labs who are trying the protocol to know what to expect. Also, was the infectivity of the sporozoites in hepatocytes determined? If yes, it could be a valuable addition to the manuscript as well.

We have added oocyst and sporozoite data from several independent experiments over the years, which shows normal variation. However sporozoite infectivity to hepatocytes is beyond the focus of this manuscript, which is primarily focused on gametocyte culture and standard membrane feeding assay.

It is not mentioned in the protocol description (Page 2, Line 121) if the feeder culture used for gametocytogenesis should be synchronous or asynchronous.

We have added more details about feeder culture, Protocol of initiating asexual stage culture (feeder culture) from a frozen stock.

Step 5 (Line 141) and Step 6 (Line 147) should reference gametocyte and exflagellation photos (should be a new figures, reorder if necessary).

Reference for gametocyte figure is included at step 7 of gametocyte culture protocol. However we do not have images of exflagellation events as we do not perform exflaflagellation assay on regular basis before blood feeding mosquitoes. Unfortunately, due to the prevailing pandemic situation it was not possible for us obtain images by performing new experiments.

The authors should mention how many exflagellation events should be expected from mature gametocyte cultures.

We have added details about the number of exflagellation needed

The ideal age of the mature female Anopheles mosquitoes used for feeding should be included in the protocol description (Page 2, Line 168).

Age of mosquito is included at the beginning of membrane feeding protocol

The authors mention on Page 4, Line 181, that the gametocyte concentration should be empirically optimized. Could they explain this in more detail?

We have described this in more detail.

Step 4 (Line 228) should reference graphic for midgut dissection (include figure, reorder if necessary).

We are thankful for the wonderful suggestion, a graphic representation of midgut dissection is added.

Typographical error on Page 5, Line 253; it should have been "Figure 2" instead of "Figure 1".

Many thanks for pointing this out, figure reference is corrected.

Typographical error on Page 6, Line 286; it should have been "temperature does not fluctuate", instead of temperature does fluctuate"

Many thanks for pointing this out, error is corrected

Figure 1: In day 1-3, leave 1 mL (to match 80%) when changing media; add 4mL, for 5mL total?; In day 4-15, daily media change, how?; show blood smear graphic; show graphic for sorting//sucrose feed post blood feed; show gametocyte, oocyst, sporozoite

We have added more details regarding media change, we leave 1-2 ml at the bottom to avoid removal of RBCs but always add 5ml fresh media every time. After media change on day 1 total culture volume increases from 5ml to 6-7 ml.

We have added data for average oocyst and sporozoite numbers, added graphics for oocyst and sporozoite dissections and sporozoite dissection protocol. Blood smear preparation is described in detail and pictures of membrane feeding set up is also included.

The labels on the x and y axis are missing in Figure 2A; check caps lock.

Labels are added in correct format

Figure 2C: It is not clear if the two midguts shown are of the same mosquito at different microscopic resolution or are of two different mosquitoes.

Images are from two different mosquitoes, description is added.

Were other mosquito growing conditions evaluated? Other temperatures tested? Humidity?

No, we have not experimented with different growing conditions for mosquitoes. But it certainly is a good idea and we can plan a separate study to evaluate this question.

Standardize spacing between units (compare Line 97 with Line 98). Standardize negative sign (Line 89 vs. Line 108)

Thank you for pointing these errors out, corrected and standardized.

On Page 2, Line 20, Giemsa-stained (see other misspellings).

Thank you for pointing these errors out, we have corrected typographical and grammatical mistakes

Fill in catalog numbers, to get exact equipment for consistency. Also, check casing (upper and lower case when appropriate).

Catalogue numbers of most reagents are added, however equipments or plasticwares can be substituted and should not have any effect on adaptation of this protocol in any laboratory.

There are some inconsistencies in the writing of units. For eg; On page 1, Line 80, there is no space between the value (20) and unit (ml), while in the same line there is space between value (30) and unit (ml). This was observed at several places throughout the manuscript.

We have standardized units throughout the manuscript.

Fix run-on sentences. For example, on Page 1, Line 70: Gametocyte producing parasite isolate: Not all culture adapted parasite lines can produce gametocytes; care should be taken while selecting parasites for gametocyte culture.

Many thanks for pointing these mistake out we have rewritten the sentence.

Reviewer #3:

Manuscript Summary:

In this manuscript, Tripathi et al., describe a detailed methodology to culture P. falciparum gametocytes in vitro and feed them to mosquitoes through standard membrane feeding assay. Few laboratories in the world have the capabilities to produce infectious gametocytes and produce mosquito infections, which emphasizes the need for the establishment of a standardized and efficient protocol. The authors are considered genuine leaders in the field of malaria transmission and the method has been fully optimized as evidenced by their multiple publications reporting successful mosquito infections with P. falciparum gametocytes. The paper is well written but has a few grammatical errors which have been mentioned below.

We are grateful to the reviewer very positive feedback

Major Concerns:

-Please provide the details of the origin of the P. falciparum NF54 used for this report. This is relevant since the authors make the point that not all P. falciparum strains produce infectious gametocytes.

Details of parasite strain (source and catalogue no) used is included in materials list

-For the exflagellation assay, it will help the reader to provide a video of an exflagellation event and/or a detailed explanation of what is an exflagellation. How many exflagellation events are expected in a healthy gametocyte culture and a productive mosquito infection?

We have added the description of the process of exflagellation and included the number of exflagellation events needed per field for successful blood feeding experiment.

-Special care must be taken when working with P. falciparum-infected mosquitoes to avoid the escape and a potential malaria transmission to humans. The authors should include in their experimental design what specific measures - secure cups, double netting, double caging - they implemented to prevent the escape of infected mosquitoes.

We have included more details about the safety measure we take with falciparum infected mosquitoes.

-Mosquito infection using standard membrane feeding assay (SMFA):
Step 7: the authors should make a statement about the care needed at this step to prevent mosquitoes from escaping. Not all facilities have a cold room inside their high-containment room and sorting unfed mosquitoes on an open space at room temperature, even when the mosquitoes are laid on top of ice, can be dangerous. In the absence of a cold room within the high-containment facility, another option is to remove the unfed mosquitoes with a mouth aspirator.

We completely agree with your observation and have added the option of using mouth aspirator to remove unfed mosquitoes.

Minor Concerns:
Mosquito mid-gut dissection and oocyst load quantification:

Step 2: For how long should the mosquitoes be immersed in ethanol to induce killing?

Usually mosquitoes are dead within couple of minutes once soaked in 70% ethanol, your point it well taken and added in the protocol.

Step 4: "stain with 0.1% mercurochrome for 2 min". Is 2 min enough time for staining? Other protocols suggest staining for 15 minutes or more. There is a discrepancy in the concentration of mercurochrome mentioned in text and Figure 1 (0.2%).

We usually 0.2% mercurochrome for 2-5 min. Protocol is updated and now concentrations are consistent throughout the manuscript.

Figures
Figure 1: The part which describes the feeding of mosquitoes can be re-drawn or replaced with an image of feeding as such.

Thank you for the wonderful suggestion we have added a new figure with pictures of feeder set up.

Figure 2A: The graph is missing the legends on both the axis.

Thank you, figure legends are added

Line 19: should read "This protocol demonstrates"
Line 42: "nature as they take 7-10 days"
Lines 47-48: "and transform into female and male gametes and begin sporogonic mosquito stage development". The way it is written give the impression that gametes goes into sporogony development which happens inside the oocyst. The authors should mention the process of fertilization, zygote, ookinete and oocyst formation which all take place before sporogony.

Thank you for pointing this out, paragraph is rewritten

Line 53: "In recent years, several methods"
Corrected
Line 85 and 95: should read µm not µM
corrected
Line 97: "add 1 ml of 500X hypoxanthine"
corrected
Line 98: "and 50 ml of pooled human serum"
corrected
Line 139: "to avoid this draw a very small volume"
Corrected
Line 113: The authors should cite the work done in P. falciparum that shows the effect of continuous in vitro passaging on gametocyte production.

Many thanks for pointing this out, we have added reference for this very important phenomenon.

Line 271: "We have described a detailed protocol"
corrected
Line 286: "making sure culture temperature does not fluctuate"
corrected
Line 287: "gametocyte development process occurs sequestered"
corrected
Please review the spacing between words throughout the manuscript.

Thank you we have diligently looked for grammatical and typographical errors.