Routine *in vitro* culture of *P. falciparum* gametocytes to evaluate novel transmission-blocking interventions

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Abstract

Preventing parasite transmission from the human host to the mosquito has been recognised as a vital tool for malaria eradication campaigns. However, transmission-blocking antimalarial drug/vaccine discovery and development is currently hampered by the expense and difficulty of producing mature *Plasmodium falciparum* gametocytes *in vitro* – the parasite stage responsible for mosquito infection. Current protocols for *P. falciparum* gametocyte culture usually require complex parasite synchronisation, addition of stimulating/inhibitory factors and may not have demonstrated the essential property of mosquito infectivity.

This protocol describes a simple and comprehensive method for culture of *P. falciparum* gametocytes that consistently mature, form gametes, and are infectious to mosquitoes. We show how functionally viable gametocytes can be used to evaluate transmission-blocking drugs both in a field setting and at high throughput for drug discovery. The protocol can be completed in 15 days, and particular emphasis is placed upon operating a gametocyte culture facility on a continuous cycle.

Introduction

Despite the huge progress made in the last 15 years, malaria is still a devastating disease causing an estimated 438,000 deaths in 2015 alone[1]. It has been increasingly appreciated that local elimination and global eradication of malaria will require the concerted effort of multiple interventions to be effective. Preventing parasite transmission from humans to the mosquito is one such intervention[2,3]. With each intraerythrocytic asexual cycle, a small proportion of parasites become committed to sexual development by an as yet unknown signal, likely triggered by host/parasite stress factors affecting epigenetic regulation and requiring the AP2G transcription factor[4–7]. Male and female gametocytes are responsible for parasite transmission to the mosquito. Peculiar to *P. falciparum* (the *Plasmodium* species responsible for most human mortality), gametocytes develop to maturity and mosquito infectiousness over ~10 days passing through five morphologically distinct stages of development (stages I-V). This prolonged period of development provides both challenges and opportunities for parasite survival. Firstly the developing gametocyte must evade clearance from the host whilst maturing. This may be achieved by sequestration in the bone marrow for a large proportion of their developmental period, only to be released into peripheral blood circulation when mature[8,9]. Secondly, in their favour, as gametocytes mature, they lose sensitivity to most antimalarial drugs used to treat disease pathology[10,11]. Unfortunately, this leads to the situation where an individual can be cured of malarial symptoms but still possess gametocytes. By this mechanism the disease, whilst treated, can still propagate throughout the population unchecked. Given that a large proportion of malarial patients will carry mosquito-infectious mature stage V gametocytes at the point of diagnosis[12], it is essential that transmission-blocking interventions target the mature, infectious, drug-insensitive stage V gametocyte to be efficacious. Another layer of complexity is added by the fact that gametocytes are sexually dimorphic, possessing both male and female gametocytes that undergo entirely different sex-specific developmental transformations during mosquito infection, and have both shared and unique biochemical pathways[13]. Male gametocytes are more susceptible to drugs than females[14], this in combination with the highly female-biased sex ratio generally observed in *Plasmodium*[15,16], raises the spectre that assays that do not include a sex-specific readout are unlikely to detect the major class of male-targeted transmission-blocking compounds.

Comparison with published methods

It has been possible to culture mosquito-infectious *P. falciparum* gametocytes since the early 1980s[17]. Generally speaking, gametocytes have been generated by maintaining asexual cultures for an extended period of time with frequent medium changes but without periodic addition of fresh erythrocytes, or sometimes reducing culture volume to raise haematocrit[17,18]. In 2007, Fivelman and colleagues reported an improved method for synchronous production of *P. falciparum* gametocytes[19]. Their protocol requires synchronising of asexual parasite stages followed by incubation of ring stage parasites with parasite-conditioned medium to artificially induce stress and trigger gametocyte induction. After sexually-committed parasites re-invade new erythrocytes, N-acetylglucosamine is added to the culture to prevent subsequent invasion events and clear the

cultures of residual asexual parasites. The Fivelman protocol has gained much popularity to date, especially by those producing large quantities of gametocytes for cell biological analysis or drug screening[10,20,21]. However, those researchers wishing to cultivate functionally viable mature gametocytes for mosquito infection studies have invariably adhered to the "early" protocol of medium replacement either by automated or manual culture[22–26].

The 'gold standard' laboratory assay for measuring transmission-blockade is the Standard Membrane Feeding assay (SMFA)[26,27]. It involves culturing gametocytes to maturity *in vitro*, introducing an intervention (e.g. drug or antibody) for a period of time, and then feeding the gametocytes to mosquitoes in an artificial membrane feeding device that simulates body temperature and presents a skin-like membrane to the mosquito. 7-10 days after feeding, mosquito infection is then assessed by quantifying the number of oocysts present in the mosquito midgut. The SMFA embraces the complex cell biology of the parasite, the complex interactions with the mosquito midgut microflora, and the immune system of the mosquito (Figure 1). Unfortunately, despite efforts to increase the throughput of the SMFA[28,29], it still remains very low throughput and at a cost prohibitively expensive for any high throughput (HTP) screening campaign. In response to this, and also to recent guidelines that transmission-blocking drugs should ideally target the gametocyte[30], many HTP gametocyte-based assays have been developed which are intended to act as a surrogate for full mosquito feeding[10,20,21,21,31–39]. Broadly speaking, most assays fall into one of two categories – "early" or "late" stage gametocyte assays. Early stage assays usually test the ability of interventions to kill stage I-II gametocytes. Late stage assays usually test the ability of interventions to kill stage IV-V gametocytes. There is some ambiguity about what constitutes an "early" or "late" stage gametocyte, with some early and late assays also including stage III gametocytes. Nevertheless, as the SMFA measures the functional viability of only stage V gametocytes (i.e. their ability to undergo further development and colonise the mosquito) and not other gametocyte stages, it is questionable how relevant assays monitoring immature stages actually are as a surrogate of transmission-blocking (Figure 1). Here we describe methods to assess the key property, the functional maturity of the stage V gametocyte – i.e. from gametocyte to gamete formation and include sex-specific readouts to identify compounds that are active on either female gametocytes or the minority male population[14,35] (Figure 1). This measurement of the functional viability of gametocytes embraces the metabolism of both the mature gametocyte and the complex pathways of gamete formation and so provides a more informative readout of transmission-blockade.

The protocol presented here allows experimenters to understand and optimise the parameters required to culture *P. falciparum* gametocytes to functional maturity in scalable quantities required for cell biological investigations and HTP drug screening. These underlying principles are then demonstrated to supply parasite biomass for transmission-blocking drug evaluation in a low throughput format suitable for research in less-specialised laboratories, and a high throughput screening assay suitable for drug discovery[14,25,34,35,40,41].

The *P. falciparum* gametocyte culture protocol described here is founded upon historical gametocyte culture protocols[17,23], however critical steps such as serum preparation, parasite strain selection, culture volumes, timings etc have been optimised and codified. At its simplest, static cultures of unsynchronised *P. falciparum* asexual parasites are continuously maintained in log phase growth by regular passaging and diluting on Mondays, Wednesdays and Fridays to maintain an uninterrupted source of parasite material for subsequent cultures. On these days, parasites are sub-cultured as required for gametocyte cultures at 1% ring stage pararasitema and 4% haematocrit (vol/vol) (HC) (Day 0). Whilst other asexual parasite stages are invariably present in the source cultures, these do not substantially affect the resultant yield of mature gametocytes; however

perfect gametocyte stage synchronisation is not achieved (see Limitations). Medium is replaced daily for 14 subsequent days without the addition of fresh erythrocytes, by which time most gametocytes have reached maturity (stage V) and are ready for experimentation. During the 15 day culture period, parasites undergo several phases of development (Figure 2) which possess different metabolic states[41]. From Days 1 – 4 after culture induction, the asexual parasitaemia increases rapidly to a peak. Stress thought to be naturally produced by the high parasitaemia induces gametocyte induction[7]. By Day 7, as the asexual population wanes, early stage gametocytes are observable. By Day 8, gametocyte maturation progresses; and by Day 14 morphologically distinguishable mature stage V male and female gametocytes are present. At this point, cultures can be harvested and used to screen for transmission-blocking drugs[35,40]. Crucially, the same protocol also generates mosquito-infectious gametocytes enabling direct validation of hits identified in transmission-blocking screens[25,42]. Additionally, the simplicity and scalability of the protocol enables the user to generate functionally viable mature gametocytes that can be used for immunofluorescence, protein samples for Western Blotting, proteomics, transcriptomics and metabolic analysis[41].

At its most basic, our strategy for transmission-blocking drug evaluation involves simulating *in vitro* as much of the cell biology of the laboratory gold standard SMFA as possible. Due to technical limitations, it is not possible to reliably support bulk *P. falciparum* ookinete/oocyst development *in vitro*, therefore male and female gamete formation demarks the furthest parasite developmental stage currently practical for incorporation into a high throughput assay. As with the SMFA, test compounds are exposed to gametocytes for 24 hr prior to induction of gamete formation, and then gamete formation is used as a readout to determine whether the compound affects the functional viability of the mature male and female gametocyte. As it is only the mature stage V gametocytes that are able to undergo gamete formation (and infect mosquitoes in the SMFA), any immature gametocyte stages that may contaminate the parasite inoculum used for the assay are not recorded. In this way, activity in our assays represent compounds that prevent transmission either by killing the gametocyte (gametocytocidal), rendering it irreversibly unable to undergo further development (sterilised) or interacting directly in the process of gamete formation (contraceptive) – meeting the requirements for a transmission-blocking drug target candidate profile recently described by the Medicines for Malaria Venture[30].

Limitations/alternative methods

This protocol is designed to yield significant quantities of functionally viable mature gametocytes. However, as unsynchronised asexual parasite feeder cultures are used to produce gametocyte cultures and gametocyte induction is triggered by endogenous culture stress factors, precisely defined gametocyte stages are not discretely present on each day, rather form a heterogeneous yet predictable population (Figure 2). Additionally, inducing gametocyte cultures at a fixed ring stage parasitaemia (which simplifies the protocol) does not take into consideration the other asexual stages present in the culture which will slightly alter the dynamics of initial asexual growth to the peak asexual population and "crash". This may cause a proportion of gametocytes to reach maturity slightly early by Days 12-13, however given the *in vivo* mean gametocyte circulation time is estimated to be 4.6-6.5 days[43], these will survive for a prolonged period and this does not affect the numbers of mature stage V gametocytes present on Day 14 of culture. For these reasons, the protocol may be unsuited to biochemical investigations requiring pure and discrete intermediate stage parasites. It is possible to sorbitol-synchronise asexual feeder cultures[19] prior to gametocyte induction to improve tightness of gametocyte development, however we have found that this

reduces subsequent gametocyte yield and functionality when they reach maturity. Similarly, it is possible to prevent further gametocyte induction by inhibiting subsequent rounds of parasite invasion by addition of N-acetylglucosamine[44] to culture medium on Day 6 onwards, however we find this can also reduce final gametocyte yields.

Experimental design

The four "pillars" of successful gametocyte culture

Four key factors affect final gametocyte yield and must be optimised by the experimenter – Parasites, Serum, Blood, and Temperature. We outline here how each factor affects gametocyte growth.

Parasites

It is commonly reported that not all *P. falciparum* parasite strains will produce gametocytes. Indeed even gametocyte-producing strains build up deleterious mutations over time in asexual culture and can rapidly lose the ability to produce mature infectious gametocytes[45,46]. Consequently the experimenter must select appropriate parasite strains for culture that are from an "early passage" (i.e. have recently completed the parasite life cycle and passed through the mosquito and back into humans). We have found the 3D7 and NF54 lab strains a useful starting point and suggest that they only be obtained from a source that confirms their mosquito-infectivity. For large-scale gametocyte culture and drug screening, NF54 is preferred as it maintains high gametocyte production for >2 months, compared to 3D7 that in our hands rapidly loses gametocyte production capabilities in as little as 2 weeks (Figure 3).

Serum

Unlike asexual parasites that can be cultured indefinitely in serum-free medium supplemented with Albumax II[47], gametocytes require human serum to optimally reach maturity[48] and virtually all mosquito transmission studies are performed with gametocytes cultured with 10% human serum[26,36]. Human serum, by nature is not a homogeneous product and shows a highly variable ability to support gametocyte growth (Figure 4). Thus each batch of serum should be tested empirically (see Box 1). Only those units able to successfully support full gametocyte maturation should be selected for final pooling to ensure a consistent supply of gametocyte-producing serum. We have found that even one "bad unit" in a pool of 24 can render the whole pool ineffective for gametocyte production. Sub-optimal serum units may be kept and used for asexual maintenance.

Blood

Blood group antigens must be compatible between blood and serum. We typically use O+ or A+ blood (whichever is available) with A+ serum. Gametocytes reside within the same erythrocyte for the duration of their 10-12 day development therefore it is advisable to use as fresh erythrocytes as possible for gametocyte induction, however storage at 4°C for up to 2 weeks does not appear to impede gametocyte culture success (Figure 5).

Temperature

Finally, the fourth critical parameter for successful gametocyte culture is temperature. Mature gametocytes must be maintained above 30°C at all times during manipulation or medium changes otherwise irreversible gamete formation is induced daily and mature gametocytes fail to accumulate[49]. To mitigate this risk, we recommend placing culture flasks/tubes that are outside of the incubator onto heater blocks maintained at 38°C at all times, minimising manipulation outside of the incubator, and using pre-warmed reagents and consumables (Figure 6).

Materials

Reagents

- Pf NF54 !Caution *P. falciparum* is a category 3 pathogen and should be only handled in approved biosafety containment with necessary local health and safety approval. Lab coat and gloves should be worn at all times and any accidental blood exposure should be reported immediately and treatment sought.
- Human Whole blood (= "RBCs") (UK National Blood Service, O+, screened for human pathogens) !Caution Although pre-screened for human pathogens, consult local health and safety regulations for handling and disposal procedures. !Caution Collection and storage of whole blood may require approval from local relevant regulatory institutions governing handling of human tissue.
- Heparin (Sigma, cat. no. H3393)
- Phosphate buffered saline (Sigma, cat. no. P4417)
- Dimethylsulfoxide (DMSO) (Sigma, cat. no. D5879)
- Gentian violet (MolPort, cat. no. MolPort-002-133-551)

- Sodium chloride (Sigma, cat. no. S3014)
- "Malaria gas": 3% O_2/5% CO_2/92% N_2 (BOC Special Gases, cat. no. 226957-L-C)

Complete culture medium (CM)

- Roswell Park Memorial Institute (RPMI) powder (to make 10 litres) (Gibco, cat. no. 13018-031)
- Sodium bicarbonate (Sigma, cat. no. S6014)
- Hypoxanthine (Sigma, cat. no. H9636)
- MilliQ dH2O
- Human serum (Interstate Blood Bank, A+ serotype no aspirin 2 hr prior to drawing, no antimalarials 2 weeks prior to drawing. Screened for common human pathogens). !Caution Even though serum is screened, it is good practice to handle with care wearing lab coat and gloves. Dispose of serum through approved waste channels.
- 5 litre conical flask (Scilabware Ltd, cat. no. 1130/36D)

Ookinete medium (OM)

- RPMI powder (to make 10 litres) (Sigma, cat. no. R4130)
- Sodium bicarbonate (Sigma, cat. no. S5761)
- Hypoxanthine (Sigma, cat. no. H9636)
- Xanthurenic acid (Sigma, cat. no. D120804)

Tissue culture consumables

- Sterile T25 flasks (Nunc, cat. no. 156340)
- Sterile T75 flasks (Nunc, cat. no. 156472)
- Sterile 1 litre bottles (for bulk culture and serum preparation) (Thermofisher, cat. no. 3110-42)
- 384 well plates (Greiner, cat. no. 781091)
- 1.5 ml tubes (Greiner, cat. no. 616201)
- 10 ml pipettes (Sarstedt, cat. no. 1 254 001)
- 25 ml pipettes (Sarstedt, cat. no. 1 685 001)
- 2 ml aspirating pipettes (SLS, cat. no. 357558)
- 50 ml tubes (Corning, cat. no. 430829)
- 15 ml tubes (Sarstedt, cat. no. 62 554 502)
- 50 ml reagent reservoirs (Sigma, cat. no. CLS4871)
- Cryovials (VWR, cat. no. BCISBCS-2511)
- 0.22 µm syringe filters (Merck Millipore, cat. no. SLGP033RB)
- 20 ml syringes (VWR, cat. no. 613-2046)

Microscopy

- Giemsa stain (Sigma, cat. no. 48900)
- Giemsa buffer - 0.7% w/v anhydrous KH_2PO_4, 1% w/v anhydrous Na_2HPO_4 in dH_2O (Sigma, cat. no. P5655, S5136)
- Methanol (Sigma, cat. no. 322415)
- Glass slides (VWR, cat. no. 631-0117)
- Fastread slides (Immune Systems, cat. no. BVS200)
- Neubauer chamber (VWR, cat. no. 720-0104)

- Anti-Pfs25 antibody 4B7 (BEI Resources (formerly MR4), cat. no. MRA-315)
- Cy3 antibody labelling kit (GE Healthcare, cat. no. PA33000)

Human serum preparation

- 290ml Buchner funnel – filter diameter 90 mm (VWR, cat. no. 511-2506)
- Whatman® GFD 90 mm diameter membrane glass microfiber (GE Healthcare, cat. no. FIL4420)
- Parafilm (VWR, cat. no. 52858-000)
- 1 litre filtering flask with glass hose connection (Duran Group, cat. no. 21 201 54)
- 500 ml 0.2 µm filter units (Thermo Scientific, cat. no. 569-0020)
- Tweezers (VWR, cat. no. 232-2115)

Equipment

- BS EN 12469:2000 compliant Class 2 Microbiological Safety Cabinet (e.g. Contained Air Solutions BioMat-2) with vacuum line.
- 37 °C Tissue culture incubator (LEEC, cat. no. C157)
- 28 °C Ultrasonic humidity cabinet (LEEC, cat. no. SFC3C/RH)
- Olympus BX43 phase contrast microscope (Olympus)
- Nikon Eclipse Ti automated microscope (Nikon)
- Pipette boy (Integra Biosciences)
- eLINE Electronic Pipette (12 channel, 50 – 1,200 µl) (Sartorius, cat. no. 730491)
- eLINE Electronic Pipette (12 channel, 10 – 300 µl) (Sartorius, cat. no. 730461)
- 3x QBD2 Dry Block Heater (Grant instruments)
- 2x Microplate Block Insert for QBD2 (Grant Instruments, cat. no QDP-FL)
- 3x 1.5 ml Microtube Block Insert for QBD2 (Grant Instruments, cat. no QB-E1)
- 3x 50 ml Tube Block Insert for QBD2 (Grant Instruments, cat. no QB-50)
- Gilson Safe Aspiration Station (Gilson)
- Microfuge (e.g. Perfect Spin Mini, Peqlab)
- Hypoxia chamber (Labquip Technologies)

Reagent Setup

Preparation of CM

Prepare 4 litre batches of incomplete medium at a time which can then be divided into convenient 200 ml aliquots and stored at -20 °C for 6 months: Add 200 mg hypoxanthine to 4 litres of dH_2O in a 5 litre sterile conical flask and stir for 3 hours to dissolve. Afterwards add 63.56 g RPMI powder and 8 g $NaHCO_3$ and allow to dissolve for a further 1 hour. Filter-sterilise through 0.2 µm filter units, prepare 200ml aliquots in T75 cm^2 flasks and store at -20 °C. CM is then prepared by adding 22 ml of human serum to 200 ml incomplete medium and can be stored at 4 °C for 1 week. CM should be at 37 °C before use and can be pre-warmed overnight. Warmed CM should be discarded after 72 hr or if it changes colour to pink (indicating pH increase).

Preparation of OM

Prepare 1 litre of ookinete medium by dissolving 1 bottle of RPMI powder, 2 g $NaHCO_3$, and 50 mg hypoxanthine into 1 litre of dH_2O in a sterile 2 litre conical flask. Prepare a 100 mM xanthurenic acid (XA) x1000 stock solution by adding 205.17 mg XA to 10 ml dH_2O. To facilitate dissolving of XA, add concentrated NaOH dropwise to the solution whilst shaking. Aliquot and store indefinitely at -20 °C. Add 1ml of XA stock solution to ookinete medium and then adjust pH to pH 7.4. Filter sterilise through 0.2 µm filter units and then store at 4 °C as 50 ml aliquots for up to 6 months.

Preparation of Heparin Stock

Dissolve heparin powder in phosphate buffered saline to achieve 300 Units/ml. Filter sterilise through 0.2 µM syringe filter and store at 4 °C for several months or -20 °C indefinitely.

Preparation of Whole Blood

Using sterile technique, aliquot donated whole blood into 25 ml aliquots and allow blood to settle. These can be stored at 4 °C for up to 2 weeks for use in cultures, although it is preferred to use blood withdrawn less than 1 week previously. For parasite culture, we have found that mixing 2.5 ml of heparin stock to the working aliquot of whole blood minimises clotting in the culture flasks.

Preparation of Gentian Violet Stock (Positive Control)

Weight an appropriate amount of gentian violet powder (e.g. ~5mg) and dissolve in a corresponding amount of DMSO to make a 10 mM stock solution. Brief shaking may be required to completely dissolve sample. Aliquot the stock solution and store at -20°C until required. Gentian violet completely inhibits the functional viability of both male and female gametocytes at concentrations >1 µM, therefore 20 µM is used as a positive control giving 100% inhibition in all screening assays. Conversely, DMSO-alone is used as a solvent carrier control in all assays to indicate 0% inhibition of male and female gametocyte functional viability.

Procedure

Continuous culture and maintenance of Pf asexual feeder cultures TIMING ongoing

This procedure describes simple maintenance of asexual feeder cultures to supply a continuous demand for gametocyte cultures. Key steps are designed to fit into the working week. The number of asexual cultures maintained is dictated by experimental demand for gametocytes and it is advisable to determine this several weeks in advance to ensure enough feeder cultures are available seeding (Figure 7).

CRITICAL Whenever cultures are outside of the incubator, they are immediately placed on a heated surface at 38 °C to minimise temperature fluctuations. A simple method to achieve this is to take a heater block and invert the metal inserts to provide a flat surface (Figure 6).

CRITICAL Whenever a culture is exposed to ambient atmosphere, it must be gassed with Malaria gas for 10 – 30 sec (dependent on culture volume) before being resealed.

1 On Monday, Wednesday and Friday (splitting days) take a 200 µl culture sample during the daily medium change (see Step 4) of feeder cultures and transfer to a 1.5 ml tube.

2 Pellet blood cells in a microfuge, remove supernatant and then prepare thin film smears and stain with Giemsa[47]. Count ring stage parasitaemia.

CRITICAL STEP Ring stage parasitaemia should be in the range 3.0 % - 4.5 %. Do not passage on culture if it is higher or lower than this as onward culture growth may stall.

?TROUBLESHOOTING

3 Assuming the feeder culture maintains 4 % HC (vol/vol), take a sample and dilute to 0.5 % (Monday and Wednesday) or 0.3 % (Friday) ring stage parasitaemia/4 % HC (vol/vol) with fresh CM and uninfected RBCs to seed ongoing asexual feeder culture(s). The remainder of the feeder culture is used to set up gametocyte cultures (Step 17).

4 On maintenance days (Tuesday, Thursday, Saturday and Sunday), whilst resting the culture on a heater block at 38 °C, carefully remove 75 % of total medium volume and discard. Bring culture back to original volume with CM warmed to 37 °C and gently resuspend culture in the fresh medium. Gas culture and return to incubator.

CRITICAL STEP All cultures require CM exchange every day for optimum growth. Ideally this should be carried out at approximately the same time each day. Extreme care is needed to prevent aspiration of cells and the maintaining of sterility.

5 Repeat previous steps continuously for up to 3 months (with NF54) with the same parasite cryovial or until gametocytes no longer required.

CRITICAL From parasite thawing to first mature gametocyte cultures takes ~ 1 month. Therefore it may be advisable to keep one asexual culture running continuously rather than repeated thawing of fresh parasite vials and prolonged waiting periods.

6 To defrost a fresh cryovial, remove from liquid nitrogen storage and thaw briefly in 37 °C water bath.

!CAUTION Liquid nitrogen causes rapid freezing on contact with living tissue and rapidly expands to displace breathable air. Appropriate training for safe handling must be completed and personal protective equipment must be worn.

7 Transfer defrosted parasites to 15 ml tube and add 0.5 volumes of sterile 12% NaCl (vol/vol) dropwise and with gentle shaking. Allow to stand for 1 min.

8 Make up to 5 ml total volume by adding 1.6% NaCl (vol/vol) dropwise whilst shaking.

9 Pellet cells at 500 g for 5 min at room temperature (~21°C to 24°C = RT).

10 Remove supernatant, make up to 9 ml with complete medium warmed to 37 °C and resuspend pellet. Repeat step 9 once more to pellet cells again.

11 Remove supernatant and resuspend pellet in 10 ml complete medium, add 400 µl fresh RBCs and transfer to a T25 cm^2 flask. Gas, seal and incubate at 37 °C undisturbed for 2-3 days.

12 Thereafter, replace medium and monitor parasite growth every 2 days by taking small culture sample and preparing a thin smear and Giemsa staining. When parasitaemia is >0.5 %, change medium daily.

13 When the culture has reached ~3.5 % parasitaemia, split culture and scale up into 2xT75 cm^2 flasks for cryopreservation and a culture for onward asexual maintenance (see Step 1).

CRITICAL STEP Samples of newly defrosted parasite lines should be cryopreserved as soon as possible to maintain good gametocyte-producing parasite stocks for the future.

14 When cultures for cryopreservation reach ~3.5 – 5.0 % parasitaemia, pellet cells in a 50 ml tube at 500g for 5 min at RT.

15 Resuspend pellet in approximately an equal volume of CM. Divide into 250 µl aliquots in 2 ml cryotubes.

16 Add 250 µl of CM + 20 % DMSO (vol/vol) to each tube, mix well and immediately snap-freeze in liquid nitrogen. Store indefinitely in liquid nitrogen storage vessel.

Gametocyte culture induction and maintenance TIMING 15 Days

17 DAY 0 of culture: Taking excess parasite material from Step 3, seed gametocyte cultures at 1 % ring stage parasitaemia/4 % HC (vol/vol). Culture volume is dependent on biomass required by experimenter (see Box 2).

18 Feed cultures by daily replacement of 75 % volume of medium along with asexual feeder cultures in Step 4 until Day 14.

CRITICAL STEP Cultures will not optimally produce mature gametocytes if a medium change is missed, especially during Days 1-7. When outside of the incubator, always place cultures on a surface heated to 38 °C.

CRITICAL STEP Do not aspirate cells during medium changes or the gametocyte culture will become progressively more dilute over time.

CRITICAL STEP When first implementing protocol, it may be helpful to smear and Giemsa stain the gametocyte cultures to monitor gametocyte growth whilst referring to expected growth shown in Figure 2.

Quantifying production of functionally viable mature stage V gametocytes TIMING 30 min

19 DAY 14 of culture: During daily medium change, resuspend settled blood cells and withdraw 200 µl of culture and place quickly into a 1.5 ml tube pre-warmed to 37 °C. Spin down in a microfuge at 2000g for 30 sec at RT, prepare a thin smear on a glass slide and stain with Giemsa.

CRITICAL STEP Giemsa slides of mature culture are important for measuring gametocytaemia and checking the “health” and development of the culture in case of troubleshooting. However, due to loss of parasite biomass, it is not advisable to take large samples from small culture volumes.

?Troubleshooting

20 For simplified quantification of exflagellation, follow option A; for accurate quantification of exflagellation, follow option B.

CRITICAL STEP The rate of exflagellation is highly temperature dependent. Room temperature assumes ~21-24 °C. Below 18 °C exflagellation will be delayed/inefficient. Above 24 °C exflagellation may occur earlier than 15 min. Above 30 °C, exflagellation may not be induced at all.

?Troubleshooting

(A) FastRead^{TM} slide method TIMING 30 min

(i) At the same time as sampling the gametocyte culture in Step 19, take another 200 µl of resuspended culture and transfer to a second 1.5ml tube prewarmed to 37 °C.

(ii) At room temperature from now on, pellet cells briefly (2000g/30 sec/RT), remove supernatant, and resuspend pellet with 10 µl ookinete medium to induce gamete formation. Set an alarm for 15 min.

(iii) Transfer parasite/ookinete mix to the chamber of a FastRead^{TM} slide and allow cells to settle.

CRITICAL STEP FastRead^{TM} slides contain eight individual chambers and so are convenient for rapid screening of multiple cultures.

(iv) At 15 min post-addition of ookinete medium, quantify exflagellation centres using phase contrast microscopy with x10 objective lens for 4 fields of view. Record the mean number of exflagellation centres.

(B) Neubauer Chamber method TIMING 30 min

(i) At the same time as sampling the gametocyte culture in Step 19, take another 10 µl of resuspended culture and transfer to a second 1.5 ml tube prewarmed to 37 °C.

(ii) At room temperature from now on, add 10 µl ookinete medium and mix to induce gamete formation. Set an alarm for 15 min and transfer to a Neubauer Chamber.

(iii) At 15 min post-addition of ookinete medium, count the number of exflagellation centres and then the RBC cell density to determine percentage of exflagellating cells in the culture (Box 3).

Exflagellation as a simple visual readout for compound transmission-blocking activity TIMING 2 days

CRITICAL This method only requires a phase contrast microscope for readout and so can be operated in non-specialised CL3 labs and is suitable for low throughput requirements (<50 observations per day).

21 Prepare enough sterile 1.5 ml tubes with 150 µl CM for each test sample and controls.

22 Add appropriate drug dilutions to tubes to ensure correct drug concentration is achieved in a total assay volume of 200 µl and with a final DMSO concentration of <0.25 % (v/v). Prepare 3x additional tubes for DMSO carrier control and 3x tubes with 20 µM Gentian Violet (final assay concentration) as a positive control. Incubate tubes at 37 °C for 15 min.

23 Take a 10 ml Day 14 gametocyte culture (Step 17) that shows at least 0.15 % exflagellating cells (Step 20B and Box 3) and concentrate cells by removing and discarding 5 ml of medium from culture.

24 Resuspend cells in the remaining medium and quickly transfer to a pre-warmed 50 ml tube.

25 Place 1.5 ml assay tubes into heater block in biosafety cabinet and quickly dispense 50 µl of resuspended, concentrated gametocyte culture to each tube. Briefly gas tubes with Malaria gas and return to 37 °C incubator for 24 hr.

26 To trigger exflagellation, take one 1.5 ml tube from the incubator and quickly remove 190 µl of culture medium whilst taking care not to disturb the settled cell pellet. Add 10 µl of ookinete

medium to tube, briefly resuspend cells and immediately transfer into a chamber of a Fastread™ slide. Start a timer for 20 min.

CRITICAL STEP If this produces a very dense cell monolayer, a larger volume of ookinete medium can be added to assist visualisation of exflagellation centres. Depending on the skill and speed of the experimenter at quantifying exflagellation, samples can be induced at staggered 5 – 10 min intervals to maximise throughput of the assay.

27 20 min post-induction, count exflagellation microscopically using x10 objective and phase contrast for 4 fields of view (Step 20A).

?Troubleshooting

28 Calculate the mean exflagellation of the DMSO controls and confirm no exflagellation centres observed in Gentian Violet positive controls. Then, calculate the percentage inhibition of exflagellation of test compounds using the following formula:

100 – ((mean test compound exflagellation / mean DMSO exflagellation) x 100) = % inhibition of test compound

384-well Dual Gamete Formation Assay for high throughput screening TIMING 3 days

CRITICAL This method requires specialised automated microscopy to record exflagellation and female gamete formation and is intended for high throughput screening.

29 Prepare 384-well plates containing test compounds dissolved in DMSO. Final assay volume will be 50 µl and final assay DMSO concentration should not exceed 0.25 %. Ensure one column contains DMSO (negative control) and one column contains a final assay concentration of 20 µM Gentian Violet (positive control). Warm plates to 37 °C in heated incubator for 30 min.

30 Take Day 14 gametocyte cultures (40 ml or greater recommended) that show at least 0.15 % exflagellating cells (Step 20B and Box 3). Pool cultures and dilute to 14 million cells per ml with pre-warmed CM to form the parasite inoculum. Allow 22 ml of inoculum per 384-well plate. Store for up to 2 hr at 37 °C until needed.

CRITICAL Ensure parasite inoculum is resuspended before use.

31 Take a 384-well plate from incubator and place on a heater block with multiwell plate attachment at 38 °C and quickly pour ~30 ml of parasite inoculum into a reservoir tray resting on a flat 38 °C heater block.

32 Working as quickly as possible, dispense 50 µl of inoculum into each well preferably using an electronic multipipette. Gently shake the plate to knock inoculum down into the well and immediately return plate to 37°C incubator and place in a sealed, humidified hypoxia chamber.

33 As needed, refill reservoir tray with inoculum and continue process until all plates are prepared and returned to the incubator. Gas plates and incubate for 24 hr. CRITICAL STEP Temperature drop will trigger gamete formation prematurely. It is essential to dispense parasite quickly and at no time pause this part of the protocol.

34 To prepare for triggering gamete formation, make up stock of ookinete medium containing anti-Pfs 25-cy3 antibody. Allow 4.5ml ookinete medium per assay plate and dilute antibody in medium to 2.7 µg/ml. Make up fresh with each assay run and store at 4 °C whilst performing assay.

35 Remove 384-well assay plate from incubator and keep a RT. Immediately add 10 µl of cold ookinete medium/antibody mix to each well using an automated multichannel pipette on the slowest dispense setting to minimise disturbing settled cells. Gently, but firmly agitate plate to drop medium down into the bottom of the wells and help mixing.

36 Immediately place plate on wellplate heater block pre-chilled to 4 °C for 4 min. Then transfer plate to wellplate heater block pre-warmed to 28 °C for a further 5 min. Keep the lid of the heater block open during the incubation.

37 Read exflagellation in automated microscope under phase contrast at x4 objective with 1.5x zoom (effectively x6) by imaging each well sequentially and recording a 10 frame timelapse image over ~1 sec.

CRITICAL STEP The timings of recording exflagellation will need to be empirically determined by experimenter to ensure that imaging occurs at peak exflagellation for the entire plate.

?Troubleshooting

38 Place test plate wrapped in aluminium foil in humidified 28 °C incubator for a further 24 hr.

39 Read female gamete formation in automated microscope using fluorescence microscopy at x6 objective by imaging each well sequentially on the TRITC channel.

40 Identify and quantify exflagellation centres and female gametes using automated analysis scripts in Icy Bioimage Analysis Program (http://icy.bioimageanalysis.org/) (Supplementary Figures 2 + 3).

41 Calculate percentage inhibition of male and female gamete formation with reference to the mean gamete counts for the negative (DMSO) and positive (Gentian Violet) control columns.

Timing

Steps 1-16: General asexual culture maintenance; ongoing continuously as required on 7 day cycle

Steps 17-18: Gametocyte culture production; 15 days

Steps 19-20: Assessing stage V gametocyte viability by exflagellation; 30 min

Steps 21-28: Low throughput exflagellation assay; 2 days

Steps 29-41: High throughput 384-well Dual Gamete Formation Assay; 3 days

Troubleshooting

Step	Problem	Possible reason	Possible solution
2	Parasitaemia too low to seed several gametocyte cultures.	Suboptimal passaging of asexual feeder cultures.	Optimise ring stage parasitaemia of asexual feeder cultures. If they are seeded too low, there will be insufficient biomass for experimentation. If they are seeded

			too high, cultures might “crash” and prematurely induce gametocyte formation.
19	Low number of gametocytes visible or mostly immature forms on Giemsa smear at Day 14.	1. Parasite line is a poor gametocyte producer. 2. Serum does not support gametocyte growth to maturity. 3. Gametocyte induction not optimal (particularly if a significant amount of asexuals remain).	1. Source a parasite line recently demonstrated to infect mosquitoes or dilution clone parasites to select for clones that may retain gametocyte production. 2. Switch serum sources. 3. Asexual parasites used to seed gametocyte cultures must be in log phase growth – do not seed from cultures with low or very high ring stage parasitaemia.
	Many round gametocytes visible on Giemsa smear at Day 14.	Premature gamete formation induced due to temperature decrease.	Ensure cultures maintain 37 °C at all times and ensure all reagents, tubes etc are prewarmed.
20	Mature stage V gametocytes are present at Day 14 by Giemsa staining, but no/low exflagellation observed.	1. Serum does not support functionally viable gametocytes. 2. Low pH of ookinete medium. 3. Gamete formation temperature not optimal.	1. See above. 2. Make a fresh batch of ookinete medium. 3. Ensure gamete formation is carried about ~21 °C. Lower temperatures will slow gamete formation. Higher temperatures will initially speed the production of gametes and >30 °C may entirely inhibit gamete formation.
27	Inconsistent/low exflagellation levels in DMSO controls.	Exflagellation is not being observed at optimum timepoint.	Ambient temperature (including heating from microscope lamp) will affect the speed at which gamete formation progresses. To determine the optimum time post-induction for readout, set up test microcultures and record exflagellation every 2 min. Repeat with several replicates and then plot exflagellation against time on a graph. The top of the curve is the optimum window for recording exflagellation. Also, very intense light sources can actually kill exflagellating gametocytes in a few seconds. Keep illumination intensity low and shutter light path when not observing cells.
37	Inconsistent/low exflagellation levels in DMSO controls or obvious exflagellation plate artefact patterns	1. Ookinete medium has not sufficiently mixed with parasites. 2. Temperature of plate has not uniformly dropped and induction of gamete formation is	1. Increase strength of shaking immediately after adding ookinete medium (Step 37) or tap plate gently against the side of a hard surface to dislodge medium. 2. Adjust the length of 4 °C and 28 °C incubations (Step 38)

		uneven.	

Anticipated Results

Using the protocol described here and an appreciation of the key factors affecting gametocyte culture, the user should expect continuous production of gametocyte cultures that consistently reach maturity by Day 14, yielding ~0.15-0.4 % exflagellating cells (See Supplementary Video 1). Gametocyte cultures showing this level of maturity and gamete formation can then be easily utilised for cell biological investigations or transmission-blocking drug research. Using a simple low throughput manual protocol, exflagellation can be used as a sensitive reporter for the functional viability of stage V male gametocytes, requiring only standard lab equipment and a basic phase contrast light microscope. Test compounds that kill the gametocyte, sterilise the gametocyte (unable to form gametes) or are contraceptive (directly inhibit gamete formation) show reduced/abolished exflagellation centres and are considered active. It is expected that DMSO control samples show exflagellation levels of ~80-100 per x10 objective field. The 384-well assay permits high throughput drug screening of the ability of compounds to affect the functional viability of both male and female gametocytes thus has both increased biological content and throughput over the manual exflagellation assay. It is expected that DMSO controls show ~180-250 exflagellation centres per x6 objective field, and ~1,600 Pfs25-positive female gametes per x6 objective field, with the male readout giving an average Z' factor of 0.54 and the female readout giving 0.91 (See Supplementary Video 1 and Supplementary Figure 1 + 2).

Box 1 - Preparation of human serum and quality control

Filtering of human serum TIMING 5 hr

1. To prepare a large homogenous batch of human serum, take 24 units (1 unit = ~200 ml).
2. In a biosafety cabinet, remove large particulate matter by prefiltering each unit through a sterile Buchner funnel containing sterile Whatman filter paper. Ensure an airtight seal on the funnel to permit effective use of vacuum. Filter paper may need to be replaced and discarded using sterile tweezers after every 50ml of serum.
3. After the prefilter, sterilize the unit of serum by passing through a 0.2 µm filter unit into a sterile 500 ml bottle. If the flow slows/stops, decant the remainder of unfiltered serum into another filter unit and continue.
4. Add 50 µl of each serum unit to separate sterile tubes containing 5ml of sterile LB medium and incubate overnight shaking at 200 rpm at 37 °C. Inspect tube for cloudiness to assess sterility.
CRITICAL STEP If serum is not sterile after test then repeat Step 3.
5. Meanwhile, assign serum units into twelve pairs. Take 10 ml from each unit and combine into pair groupings of 20 ml total. Aliquot into 5x4 ml and store at -20 °C until needed. Store the rest of the serum at -20 °C.

Serum unit quality control TIMING 15 days

6. Set up twelve individual gametocyte cultures in T25 cm^2 flasks (See Step 7 of main protocol) and maintain exclusively with CM containing serum from each of the serum pairs. Also set up a control culture with serum from pre-existing (good) serum batch for comparison.
7. On Day 14, quantify exflagellation (See Step 21 of main protocol) and compare to control culture. Reject those serum pairs that do not support exflagellation.
CRITICAL STEP If a serum pair does not support exflagellation, the units can be retained and re-tested individually with subsequent serum preparations to identify the inactive unit.
8. Defrost all serum samples that support exflagellation and pool in multiple 1 litre Biotainer Bottles. Aliquot into 22ml aliquots and store at -80°C for up to 1 year.

Box 2 – Scalable gametocyte cultures

Culture Volume	Vessel used	Notes
0.2 ml	96-well plate	Care must be taken to prevent aspiration of cells. A humidified environment is essential to minimise evaporation.
2 ml	6-well plate	Care must be taken to prevent aspiration of cells. A humidified environment is essential to minimise evaporation.
10 ml	T25 cm^2 flask	Appropriate volume for low throughput assays.
40 ml	T75 cm^2 flask	Appropriate volume for high throughput assays.
200 ml	1 litre Bottle	Appropriate volume for high throughput assays.

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Box 3 – Viability measurements: Gametocytaemia vs Exflagellation

Conventionally, gametocytaemia is often reported as a percentage of total erythrocytes by microscopic observation from thin smear Giemsa stained slides. This does not reliably report total gametocyte numbers, as cultures undergo variable amounts of RBC lysis as culture progresses which can falsely inflate the relative gametocytaemia. Also, it relies on qualitative assessment of gametocyte maturity by relative cell morphology which does not report of the functionality of the gametocyte. Measuring gamete formation is far superior as only fully mature gametocytes are able to form gametes, therefore culture functionality can be directly measured. Although only measuring male gametocytes, exflagellation is a convenient readout for culture success due to its speed of progression, and simplicity of observing by light microscopy. In our experience, males are a more sensitive readout than females and we are yet to observe a wild-type culture that exflagellates but does not form female gametes.

Quantifying exflagellation TIMING 25 min

1. 15 min after taking a culture sample and diluting 1:1 (vol/vol) in ookinete medium and introducing to a Neubauer Chamber, observe exflagellation by phase contrast microscopy at x40 objective.
2. Count and record the number of exflagellation centres in the four 4x4 outer grids of the Neubauer Chamber (Figure 8).

CRITICAL STEP Ensure that only exflagellation centres are recorded and not free gametes. Each male gametocyte produces up to eight gametes and so counting gametes will lead to overestimation of exflagellation. At high RBC densities, exflagellation centres may appear to be localised sites of disturbance of the RBC monolayer. At low RBC densities, exflagellation centres show a small central mass with multiple flagella rapidly flexing out from the centre.

3. Calculate exflagellation centres per ml of culture using the following formula:

mean exflagellation of 4 grids x 2 (dilution factor) x 10^4 = culture exflagellation per ml

4. Count phase-bright RBCs in sixteen small squares of the central grid and calculate the number of RBCs per ml of culture using the following formula:

mean RBCS of 16 small squares x 100 x 2 (dilution factor) x 10^4 = RBCs per ml

5. Use the output of Steps 3 and 4 to calculate the number of exflagellating cells as a percentage of total cells:

(culture exflagellation per ml / RBCs per ml) x 100 = % exflagellating cells

7. The parameters calculated in Steps 4 and 5 are useful for determining whether functionally viable gametocyte production is adequate for subsequent bioassays.

Anticipated Results
A successful mature gametocyte culture generally possesses ~0.15 % to ~0.40 % exflagellating cells within a RBC density of ~40 million to ~90 million RBCs per ml. If this is not obtained, see troubleshooting section.

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Authorship

MJD, US, AR, CMB and SM all contributed to development and standardisation of the protocol. US provided parasite culture support and supplied primary data for manuscript. JB and RES guided the process. MJD compiled and analysed the primary data and wrote the draft manuscript with all authors contributing to the final manuscript.

Competing Interests

The authors' declare that they have no competing financial interests.

Figure Legends

Figure 1 – *P. falciparum* transmission stage development from onset of gametocyte development in the human host to oocyst formation in the mosquito compared to the parasite cell biology included in key classes of transmission-blocking assays.

Figure 2 – The developmental progression of gametocyte cultures using this protocol. Images and data modified from Lamour *et al*[41]. Black scale bar indicates 10 µM; white scale bar indicates 150 µm.

Figure 3 – Historical culture data showing how the efficiency of functionally mature gametocyte production declines for NF54 and 3D7 strain *P. falciparum* parasites the longer a parasite line is kept in continuous asexual culture. Single cryovials were thawed from parasites recently passed through the entire parasite life-cycle (thus initially are able to generate gametocytes) and were continuously cultured as asexuals until their ability to generate gametocyte cultures was no longer possible or the experiment ceased. Gametocyte cultures were regularly seeded from these parasite lines at different timepoints and culture viability measured by assessing exflagellation on Day 14 of culture induction. If multiple cultures were set up on the same day, the mean exflagellation was calculated. n = 72 cultures (NF54) and 20 cultures (3D7). Trendlines show exponential curve fitting.

Figure 4 – Individual units of human serum show great diversity in appearance and the ability to culture *P. falciparum* gametocytes to maturity.

Figure 5 – Pooled data for historical gametocyte cultures induced with blood stored for a varying time at 4 °C after withdrawal. Datapoints indicate the mean and error bars denote the standard error.

Figure 6 – A typical arrangement of a biosafety cabinet for routine P. falciparum gametocyte culture.

Figure 7 – The protocol workflow relies on continuous culture of asexual feeder cultures that are periodically split and excess parasite biomass then used to set up gametocyte cultures. Gametocytes develop to maturity over a further 14 days with daily medium replacement whilst maintaining a constant temperature of 37 °C.

Figure 8 – Areas of Neubauer Chamber to count to accurate calculate exflagellation. Green squares indicate four areas to count exflagellations and blue squares indicate four areas to count RBC numbers.

Supplementary Information

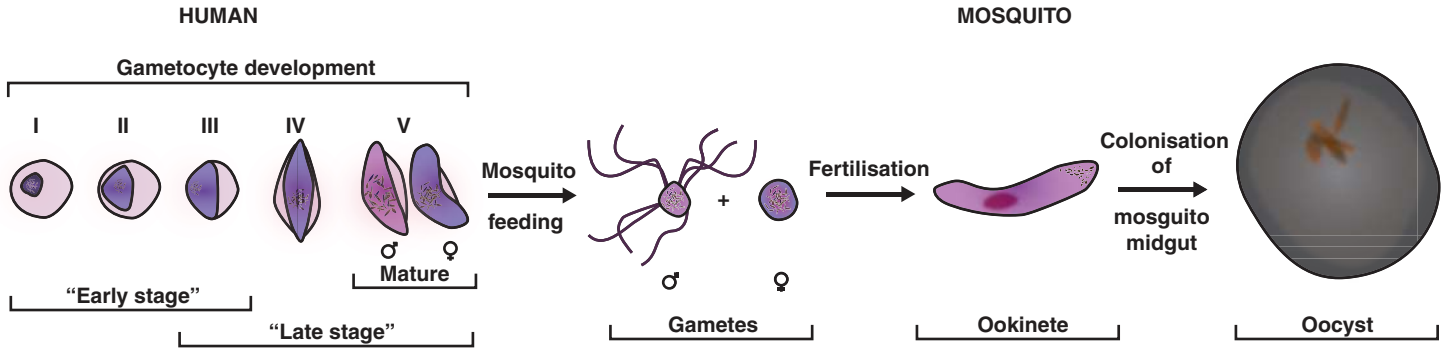
Supplementary Video 1 - Two exflagellating cells (red arrows) 20 min-post induction, imaged at x100 objective with differential interference contrast filters.

Supplementary Video 2 - Enlarged area of x6 objective movie of exflagellation centres (red arrows) illustrating the expected results required to detect exflagellation under assay conditions described in this protocol.

Supplementary Figure 1 – Exflagellation is detected and quantified from x6 objective 10 frame timelapse images. Exflagellation centres are visualised indirectly as areas of disturbance of the RBC monolayer that can be identified using ICY Bioimage Analysis program (http://icy.bioimageanalysis.org/). Scale bar = 1000 µm.

Supplementary Figure 2 – Female gamete formation is detected and quantified from x6 fluorescence microscopy images. Female gametes are visualised by live staining with Cy3-conjugated anti-Pfs-25 antibody and identified using ICY Bioimage Analysis program (http://icy.bioimageanalysis.org/). Scale bar = 1000 µm.

TRANSMISSION CELL BIOLOGY



GOLD STANDARD LABORATORY ASSAY

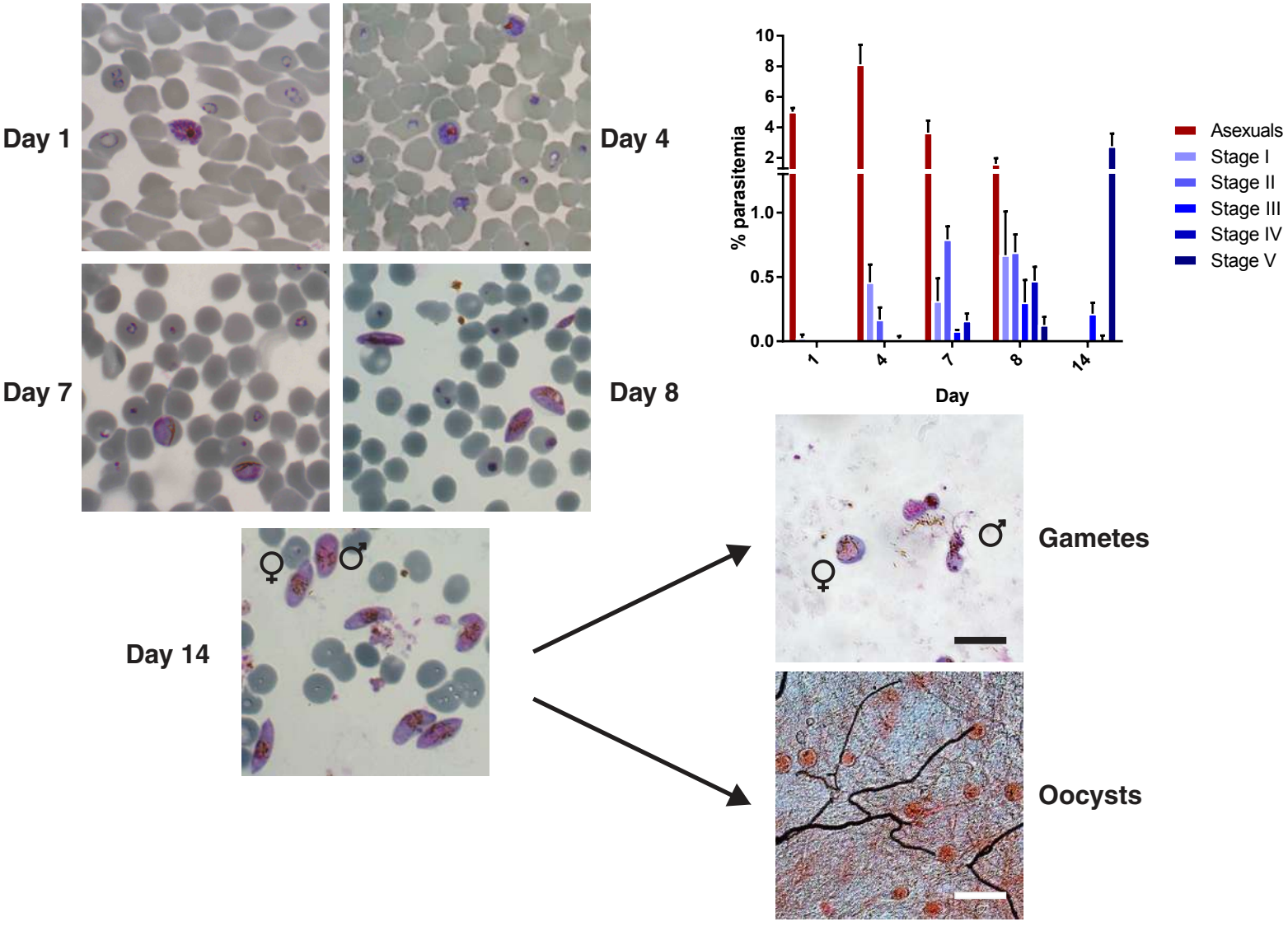
Standard Membrane Feeding Assay (SMFA)

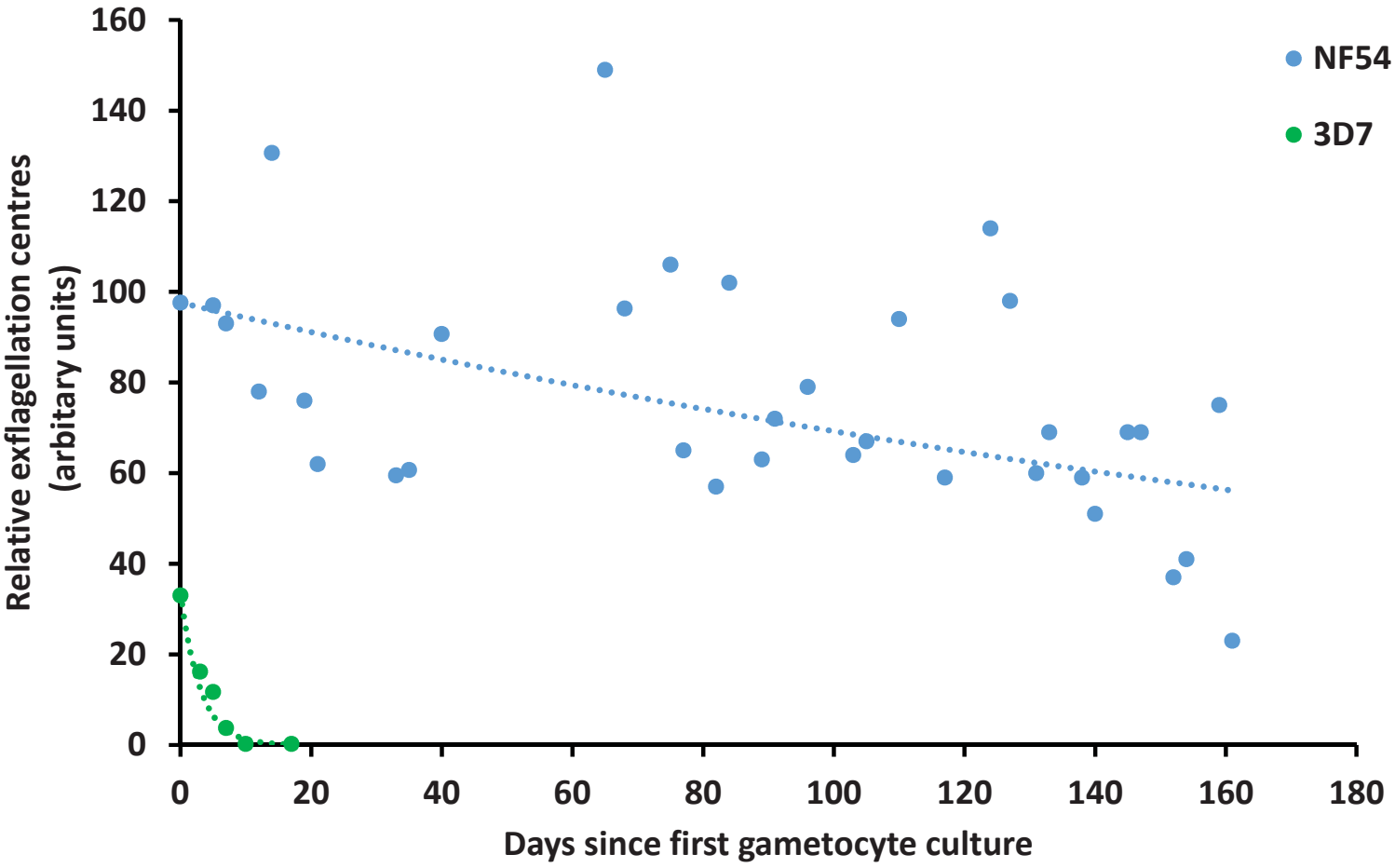
Early gametocyte assays

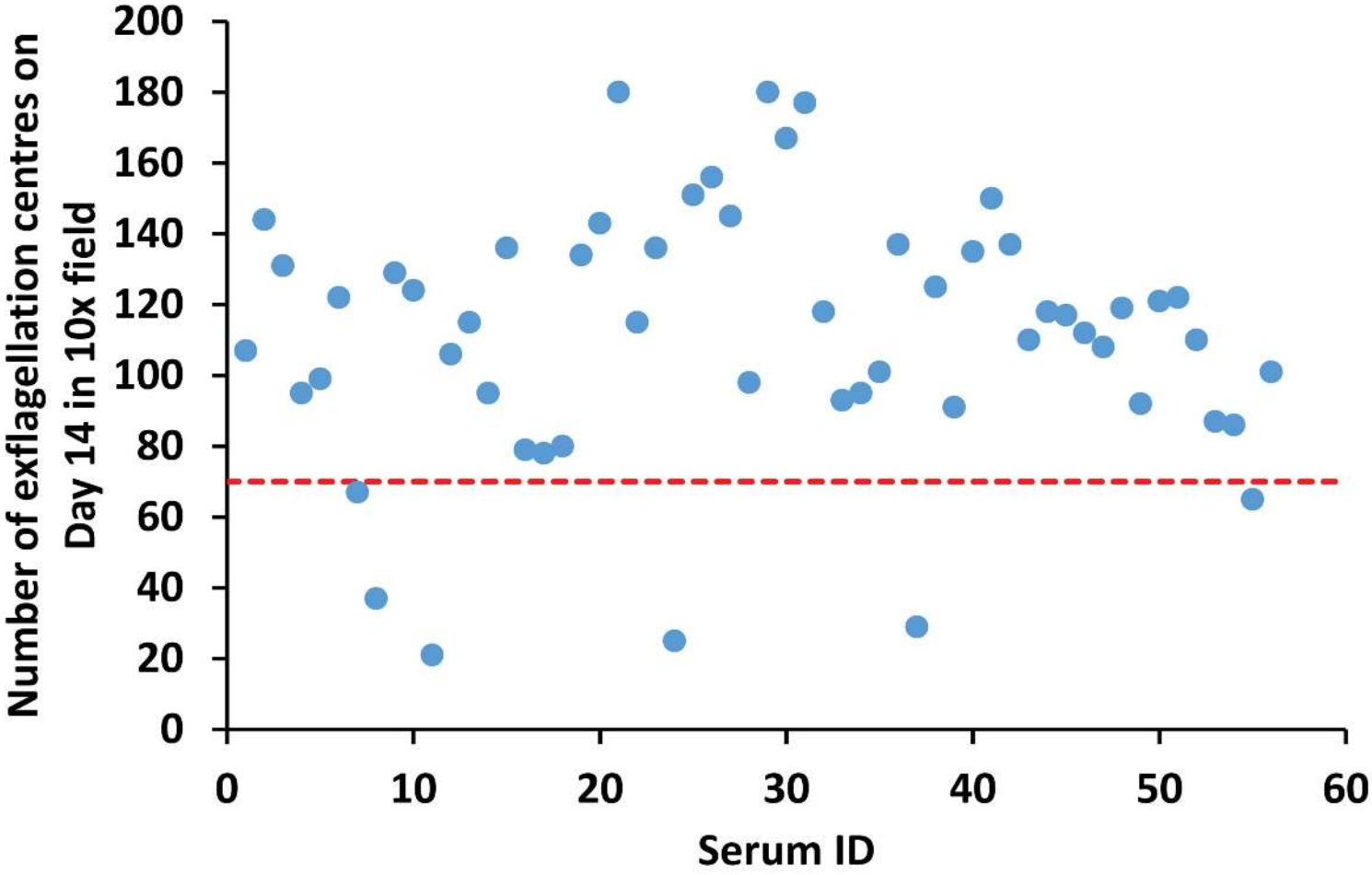
Late gametocyte assays

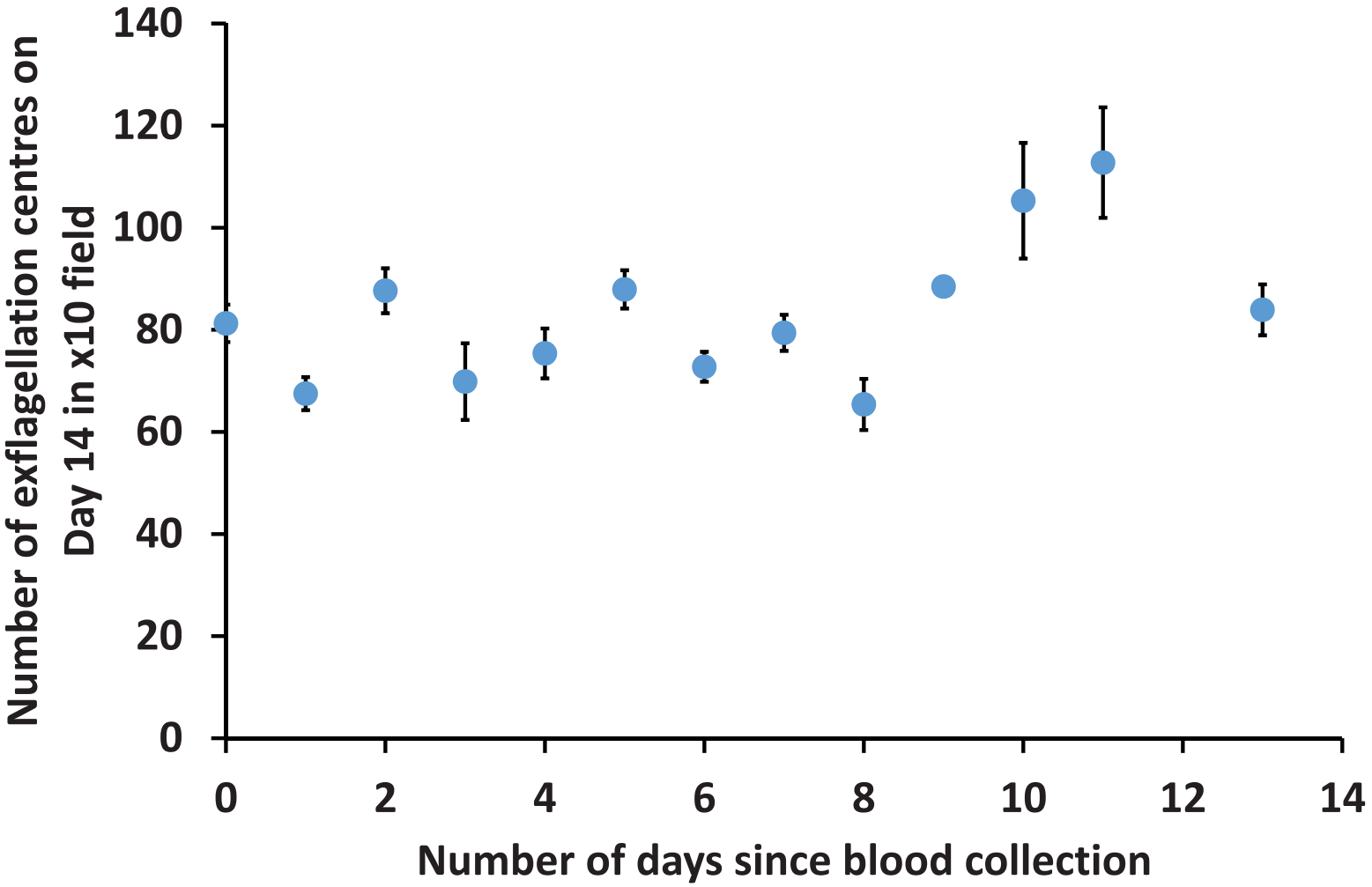
Gamete formation assays

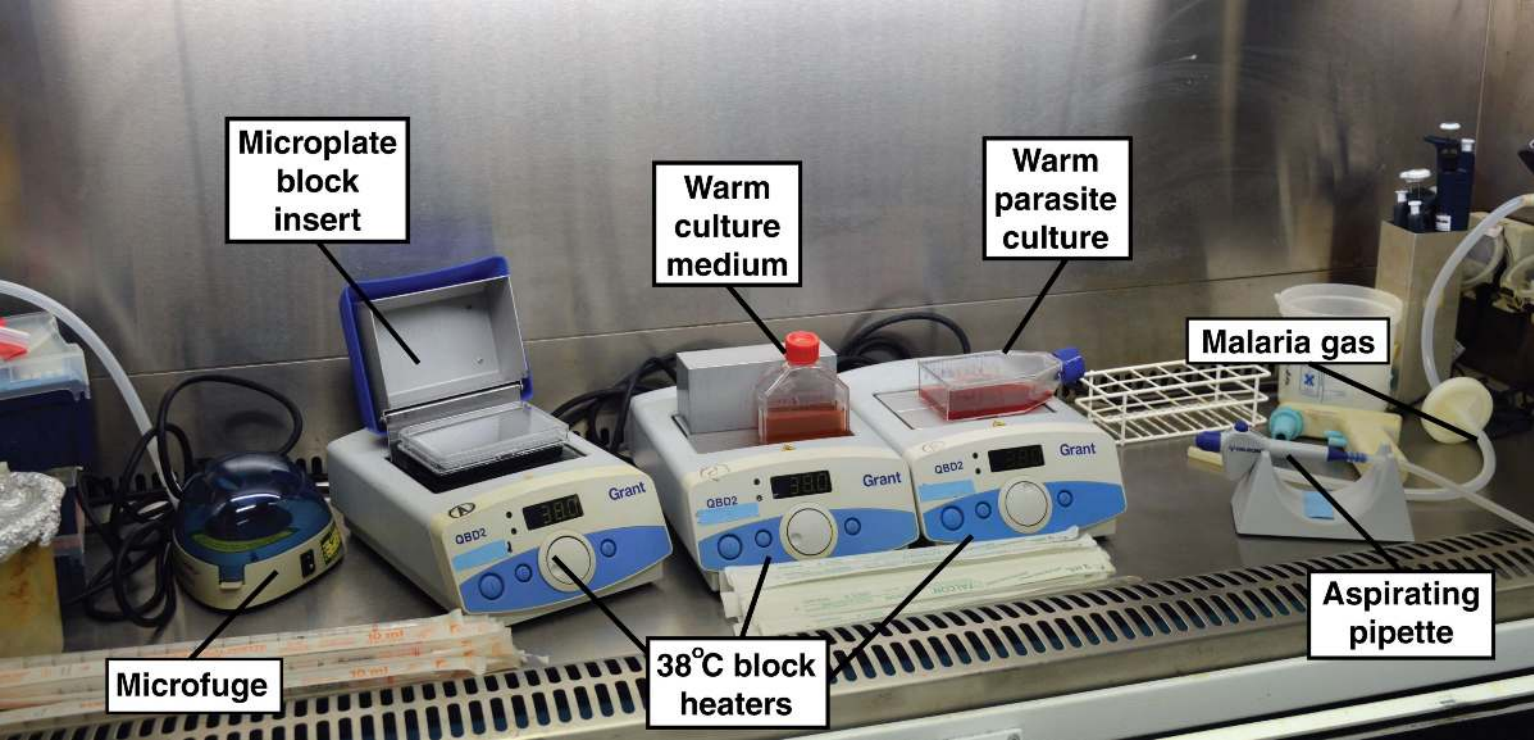
TRANSMISSION-BLOCKING ASSAYS

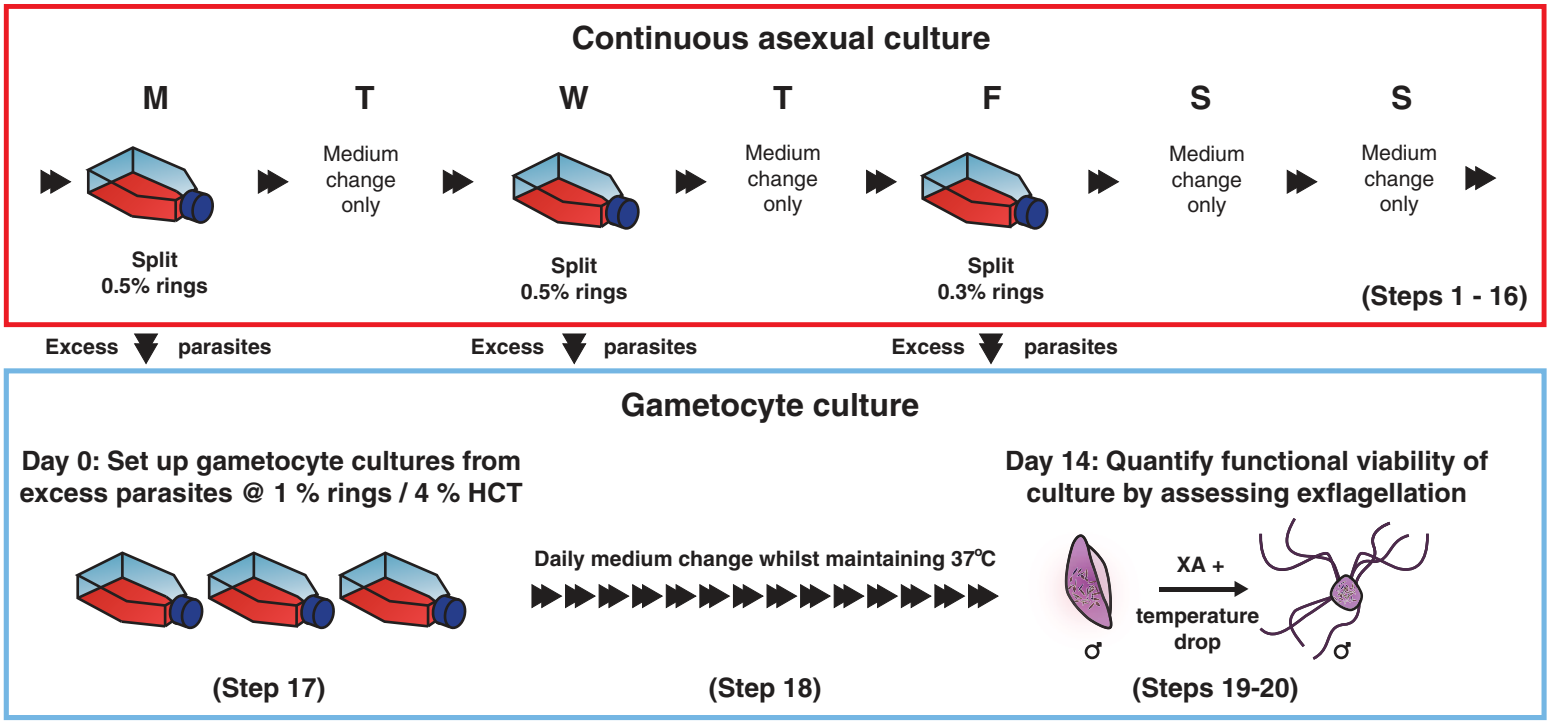

Day 1
Day 4
Day 7
Day 8
Day 14
% parasitemia
10
8
6
4
2
1.0
0.5
0.0
1
4
7
8
14
Day
Asexuals
Stage I
Stage II
Stage III
Stage IV
Stage V
♀
♂
Gametes
Oocysts

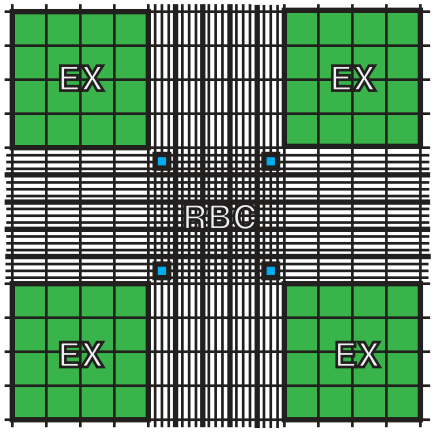

Relative exflagellation centres (arbitary units)
160
140
120
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
180
Days since first gametocyte culture
NF54
3D7


Number of exflagellation centres on Day 14 in 10x field
200
180
160
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
Serum ID


Number of exflagellation centres on Day 14 in x10 field
140
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Number of days since blood collection


Microplate block insert
Warm culture medium
Warm parasite culture
Malaria gas
Aspirating pipette
Microfuge
38°C block heaters
Grant
QBD2


Continuous asexual culture
M
T
W
T
F
S
S
Medium change only
Medium change only
Medium change only
Medium change only
Split 0.5% rings
Split 0.5% rings
Split 0.3% rings
(Steps 1 - 16)
Excess parasites
Excess parasites
Excess parasites
Gametocyte culture
Day 0: Set up gametocyte cultures from excess parasites @ 1 % rings / 4 % HCT
Daily medium change whilst maintaining 37°C
Day 14: Quantify functional viability of culture by assessing exflagellation
XA + temperature drop
♂
♂
(Step 17)
(Step 18)
(Steps 19-20)


EX
EX
RBC
EX
EX