Full Length Research Paper

Development of a Robust and Reproducible Method for the *In Vitro* Cultivation of *Plasmodium falciparum* Gametocytes for Mosquito Infectivity Studies

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In vitro gametocytogenesis is a prerequisite for studying the sexual stages of the human malaria parasite *Plasmodium falciparum*. Procedures for the high-yield production of *P. falciparum* gametocytes stages I to V, and for artificially triggering male gametogenesis which is the next step of the life cycle inside the mosquito vector, are described. Gametocytes of *P. falciparum* are established from continuously maintained cultures of asexual blood stage parasites. In order for gametocytes to comprise a substantial proportion of parasitized erythrocytes, it is necessary for asexual stage parasites to reach a high density and become stressed *in vitro* before conversion to gametocyte production. This occurs typically 6-8 days after the dilution of a culture. Gametocytes then require at least a further 8 days to reach maturity. Cultures for gametocyte production are therefore maintained for a minimum of 14 days without further dilution with fresh uninfected erythrocytes in order to obtain a large proportion of mature and infectious gametocytes. We routinely perform gametocyte feeds to mosquitoes using cultures propagated continuously for the preceding 14-17 days. Here, a robust and reliable method for production of high levels of infectious gametocytes is described. This is evidenced by the demonstration of the *in vitro* transformation of male gametocytes into gametes by the process of exflagellation.

INTRODUCTION

The most deadly of all tropical diseases, malaria kills an estimated 2-3 million people annually (Murray *et al.*, 2012). The spread of resistance to drugs of the aetiological agent, protozoan *Plasmodium* parasites, and to insecticides of the vector, *Anopheles* mosquitoes, has made obsolete various tools for malaria control. This intractable problem has stimulated new approaches to the control of both transmission of the parasite and the impact of infection (Greenwood, 2008). Contributing significantly to the lack of success is the relative difficulty encountered in culturing *in vitro* the blood-borne stages of the *Plasmodium* life cycle. This

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hinders fundamental research in cell biology, biochemistry, immune evasion, immune mechanisms, susceptibility and resistance to candidate drugs, that underpins vaccine design and developments in chemotherapy (LeRoux *et al.*, 2009).

Asexual proliferation of malaria parasites proceeds by cycles of intraerythrocytic multiplication, rupture and reinvasion. With each cycle a small proportion of invasive stage parasites, merozoites, undergo terminal differentiation to become gametocytes, the sexual stages infective to the mosquito (Dixon *et al.*, 2008). Once a sexually committed merozoite invades an erythrocyte, it begins a complex differentiation and maturation process. It starts as a stage I gametocyte that is morphologically almost identical to an asexual trophozoite and ends with the formation of a large crescent-shaped stage V gametocyte.

The mature gametocyte is the only form of the parasite that is able to survive in the midgut of the mosquito vector, an inhospitable environment it finds itself following uptake in a blood meal (Baton and Ranford-Cartwright, 2005). Thus, understanding gametocytogenesis may facilitate improved strategies for malaria control. Nonetheless, producing high yields of gametocytes *in vitro* is not without difficulty. We have developed a detailed protocol for the reliable production of large numbers of gametocytes of *P. falciparum*. This will benefit considerably the study of each stage *in vitro* and of mosquito infectivity of mature gametocytes *in vivo*.

MATERIAL AND METHODS

Safety Precautions

P. falciparum is a highly dangerous human pathogen which is classified internationally as requiring biosafety level 3 containment (Centers for Disease Control and Prevention, 2009). Hence, all appropriate health and safety measures must be carried out when maintaining this parasite in human blood *in vitro*, in keeping with local regulations and institutional guidelines. Any isolate of *P. falciparum* that is used routinely should not be resistant to standard antimalarial drugs. All work should be performed in a Class II safety cabinet and all non-essential staff should be excluded from the laboratory while work is in progress. A Howie-style laboratory coat and latex gloves should be worn at all times.

Care should be taken in the disposal of all potentially infective materials, such as blood, serum and parasites, following local guidelines. Typically, spent medium and all used disposable plastic ware should be discarded in a hypochlorite disinfectant (e.g. Chloros or Presept) prepared to a concentration of 10% free chlorine prior to disposal. All working areas should be swabbed with 70% ethanol at the conclusion of any work. A wash bottle of 70% ethanol should be readily available to flood any spillage of infective material.

Human blood and serum should be obtained from reliable sources only, such as a recognised local blood transfusion or banking service, where blood products are routinely pre-screened for the presence of HIV and hepatitis viruses.

Sterile Plastic Ware

There are many reputable international suppliers of plastic ware for tissue culture. For culture of *P. falciparum in vitro*, 25cm^2 cell culture flasks (Iwaki 3100-025 or similar) and 30 ml Universal containers (Sterilin 128A or similar) should be used. Medium should be dispensed or transferred using graduated plastic pipettes; these should be singly wrapped and disposable (2, 5 and 10 ml; Sterilin or similar).

Preparation Of Complete Culture Medium

RPMI 1640 medium is purchased as sterile 1 litre bottles (Gibco, 041-91187A). This medium contains 5.96 g/L HEPES buffer, 3.60 g/l glucose, and 50.0 g/l hypoxanthine. To each 1 litre bottle is added 42 ml of freshly prepared filter-sterilized 5% sodium bicarbonate (Sigma S-5761); this is referred to as 'incomplete' medium. Following the further addition of 100 ml of heat-inactivated pooled human serum, the liquid is now termed 'complete' medium. This should be stored at 4 °C and used within 7 d, during which time the pH should be monitored (optimum pH 7.3).

Parasites

In order to maximize the likelihood of satisfactory gametocyte production, it is essential to use a line or clone of *P. falciparum* for which production of gametocytes is established. Lines well known for gametocyte production include NF54, 3D7A and HB3A, each of which is obtainable at no cost to registered researchers from a central resource for malaria reagents (MR4, 2014).

Cultures should be restarted from frozen stocks every 2 months or less since gametocytogenesis in a continuously cultured line will start to wane after this period. There is a need to monitor and record gametocyte numbers in order to determine when a new stabilate is needed. This enables overlapping of cultures from successive stabilates so that parasite cultivation is a continuous, not a start/stop, process.

Retrieval Of Parasites From Liquid Nitrogen

The cryotube containing parasite stabilate should be placed in a 37 °C water bath for 2 min. The thawed content is then transferred to a sterile microcentrifuge tube and spun at 10,000 × *g* for 1 min. After removing the supernatant, the pellet is resuspended in 1 ml of PBS + 10% sorbitol; this should be added slowly, drop-wise, with continuous mixing. The washing procedure should be repeated twice. Following the third and final wash, once the pellet is resuspended in complete medium the cells are ready for culture.

Selection And Preparation Of Human Erythrocytes

Group O, Rhesus group-positive whole blood is preferred, and this should be less than 7 d old. After transfer into adenine-CPD (citrate phosphate dextrose),

blood is centrifuge at 800 × *g* for 5 min, then white blood cells removed by aspiration of the buffy coat. An equal volume of incomplete medium is added and centrifuged at 800 × *g* for 5 min before discarding the supernatant. This step is repeated twice, i.e. a total of three washes. Following washing, the packed erythrocytes may be used directly (i.e. at a 100% haematocrit) and any remainder discarded. Alternatively, the packed erythrocytes may be resuspended in an equal volume of complete medium (i.e. at a 50% haematocrit), which should be stored at 4 °C and used within 7 d. Keeping erythrocytes in complete medium (with human serum) greatly enhances their viability and prevents lysing during storage.

Selection And Preparation Of Human Serum

Human group O, Rhesus group-positive serum should be pooled from a minimum of 10 donors to minimize variations of quality between individuals. Packs of serum are routinely heat-inactivated at the source, but if not they should be incubated at 56 °C for 1 h. Before full-scale use, each pool should be tested to ensure that it is sterile and supports parasite growth, in particular that of gametocytes. Aliquots may be stored at −20 °C for up to 6 months.

Gas Mixture

The gas mixture of choice is 1% oxygen, 3% carbon dioxide and 96% nitrogen (BOC Specialty Gases, or equivalent local specialist gas supplier). This should be passed from the cylinder via a gas-reducing valve into the safety cabinet using a silicon tube. The gas is filtered through an in-line Whatman gamma 12 sterilizing unit with a 0.3 µm filter tube. The filter is connected to a sterile Millex GS 0.22-µm disc filter, the end of which is attached to a 19G × 1.4 inch blunt-ended needle fitted to a 1 ml syringe. When gassing a culture flask, the flow rate should be moderate but sufficiently strong to ruffle the surface of the liquid.

Preparation, Staining and Viewing of Thin Blood Films

It is recommended to use improved R66 Giemsa (BDH 350864X) diluted 1:10 with Sorensen's buffer (HD Supplies; HDS 20, pH 7.2) (Read and Hyde, 1993). A small volume of suspended erythrocytes should be removed from the culture flask and smeared across a glass slide to give a monolayer of erythrocytes. This thin blood film should be air-dried, fixed in 100% methanol and stained for 20 min in the 10% Giemsa solution. Glass slides should be washed with tap water for 20 s and allowed to air dry before viewing by light microscopy using oil immersion at 1000 × magnification.

Propagation Of Asexual Blood Cultures

The 3D7A cloned line of *P. falciparum* may be cultured routinely and is, in our experience, a reliable producer of gametocytes. Hence, it is recommended for successful gametocyte cultivation (Graves *et al.*, 1984). Whichever line is used, an isolate should be retrieved from liquid nitrogen and placed in a 25 cm^2 tissue culture flask along with 0.25 ml of freshly washed erythrocytes and 6 ml of complete medium. The culture is gassed for 30 s and the culture flask cap screwed on until finger-tight. This is then kept in a dedicated 37 °C dry incubator.

At the same time each day the culture requires a medium change. The flask is tipped at 30 degrees to the horizontal, which results in good separation between erythrocytes and the medium that forms the over-layer. The use of a specially designed autoclavable tray allows the cultures to be inclined at 30 degrees and to be moved easily from incubator to culture hood without disturbing the settling cultures. As flasks are settled, the appropriate volume of complete medium should be warmed to 37 °C. Settling of flasks and warming of medium takes 40-60 min. Spent medium is then removed from each culture, discarded and replaced with an equal volume (6 ml) of fresh medium, and the flask gassed for 30 s.

All operations should be performed on a warming plate (permanently kept in the safety cabinet) set to 37 °C in order to minimize heat loss to cultures during the time they are out of the incubator. It is important to maintain cultures and to keep all equipment as close as possible to 37 °C, since an unregulated drop in temperature may trigger gametocytogenesis before it is desired. To this end, routine, recorded checks of the temperature of the incubator and warming plate should be carried out.

Growth of Blood Cultures for Gametocyte Production

In order to produce gametocytes on a long term basis, it is necessary to maintain a stock of 'asexual' cultures from which the gametocyte cultures are drawn. Every 5 d, flasks which have reached or exceeded a parasitaemia of 1% may be subcultured ("split") by adding fresh erythrocytes in order to reduce the parasitaemia to about 0.5%. In this way, from one flask at a high parasitaemia a number of 'daughter' flasks may be started at a lower parasitaemia. Some of these flasks may be used for further stock asexual cultures and some may be used for gametocyte culture.

The new asexual stock cultures are incubated for another 5 d before being split again when a minimum parasitaemia of 1% is reached. By this means, a continuous supply of cultures for later gametocyte preparation is guaranteed. Existing gametocyte cultures should not be subcultured but instead continue to have their medium changed daily. Progress can be checked by examining thin blood films taken periodically post-subculture, typically on days 8, 12 and 15.

Experience shows that attempts to cultivate gametocytes in large numbers can cause diminished returns. Traditionally, gametocyte cultures have only

been maintained with human serum; however, it was reported recently that 1% AlbuMax II (Invitrogen) can also support the growth of gametocytes (Buchholz *et al.*, 2011).

Although it is possible to attain much higher gametocytaemias than those attained here by enriching for gametocytes by a variety of treatments (Percoll or Nycodenz density gradient centrifugation or selective drug treatment are the most commonly used), this is not recommended for mosquito infectivity studies. In the experience of ourselves and others (Saliba and Jacobs-Lorena, 2013), artificially increasing the gametocytaemia is often counterproductive to satisfactory transmission of the parasite to the mosquito vector. However, if large numbers of trophozoites and schizonts are present with late stage gametocytes, the cultures may be treated with 5% D-sorbitol. This will kill the trophozoites and schizonts but not the gametocytes.

Development of male sexual stage parasites is more rapid than that of females, so mature male gametocytes may be seen from 14 d but mature females usually appear only from 16 d post-subculture. In order to ensure a satisfactory balance of male and female gametocytes for the purposes of transmission, it is common practice to mix cultures of different ages at the time of the blood feed.

In Vitro Examination of Male Gametogenesis

Exflagellation is the explosive production of male gametes of the malaria parasite, which happens in the mosquito midgut within a few minutes of a blood meal. This phenomenon also occurs spontaneously *in vitro* and thus may be observed in cultures of fresh parasitized blood under the light microscope (1000× magnification under oil immersion). It is controlled *in vitro* solely by the change from 37 °C to the ambient laboratory temperature, the pH rise this brings being mediated by a fall in CO_2 tension as the blood equilibrates with the atmosphere (Carter and Nijhout 1977).

Viewing of gametogenesis (typically between 10-25 min after removal of a blood culture from the 37 °C incubator) is a strong indicator that oocysts and sporozoites will be produced following a blood feed. However, although it is a frequent and reassuring sight, in our experience observable exflagellation is not a prerequisite for successful mosquito infectivity.

RESULTS

By following this methodology with *in vitro* cultures of *P. falciparum*, efficient rates of transformation of asexual blood stage parasites to form gametocytes, then gametes, may be achieved. Developmental stage I and II gametocytes will start to appear after 6-8 d post-subculture, and, typically, stage III gametocytes after 8-10d (following asexual stage 'stress', which occurs once a threshold parasitaemia is reached) (Figure 1).

Stage IV gametocytes may be observed over the following week (Figure 2). From 14 d post-subculture flasks should contain mature, stage IV gametocytes (Figure 3). By 17 d, 1-4% of parasitized erythrocytes should be stage V gametocytes and the gametocytaemia of all stages of development (I-V) may be up to 12%. Progress can be checked by examining thin blood films taken periodically post-subculture, typically on days 8, 12 and 15.

For gametogenesis, two distinct developmental stages may be recorded, the morphological change of 'rounding up' from a crescent to a spherical gametocyte that is undergone by both male and female cells, followed rapidly by exflagellation of males only. During exflagellation the cytoplasm of the male gametocyte becomes agitated, followed rapidly by the sudden and vigorous protrusion and detachment of up to eight flagellated gametes (spermatozoa) from the surface of the parasite cell body (Figure 4).

In our experience (Taylor-Robinson and Looker, 1998), approximately 40-47% of stage V gametocytes will 'round up' within 21 minutes of removal of a culture flask from 37 °C to the ambient laboratory temperature. Exflagellation is observed in 20-23% of the same cells after a further three minutes. For individual gametocytes, the entire process of rounding up and exflagellation takes less than a minute.

CONCLUSION

The production of gametocytes of *P. falciparum in vitro* is an exacting and challenging task. The methodology that is described provides consistently high yields of stage V gametocytes that are capable of undergoing gametogenesis and surviving in the mosquito vector. This enables improved studies of the transformation between parasite development stages *in vitro* as well as to facilitate an enhanced efficiency of *in vivo* mosquito infectivity.

### ACKNOWLEDGEMENTS

CONFLICT OF INTEREST

None is declared.

AUTHORS' CONTRIBUTIONS

M. Looker performed *in vitro* cultivation of *P. falciparum* asexual blood stages, growth for gametocyte production, and examination of gametogenesis. A.W. Taylor-Robinson supervised execution of the work and helped with *in vitro* parasite cultivation and observation of exflagellation. Both authors conceived the design of the study, processed and analysed the data, prepared the manuscript and approved the final version.

REFERENCES

Baton LA. and Ranford-Cartwright LC (2005). Spreading the seeds of million-murdering death: metamorphoses of malaria in the mosquito. Trends Parasitol. 21: 573-580.

Buchholz K, Burke TA, Williamson KC, Wiegand RC, Wirth DF and Marti M (2011). A high-throughput screen targeting malaria transmission stages opens new avenues for drug development. J. Infect. Dis. 203: 1445-1453.

Carter R and Nijhout M.M (1977). Control of gamete formation (exflagellation) in malaria parasites. Science 195: 407-409.

Centers for Disease Control and Prevention (2009). Biosafety in Microbiological and Biomedical Laboratories, 5th edn. US Department of Health and Human Services, Bethesda, MD, USA. Retrieved September 3, 2014. Available online at:
http://www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf

Dixon MW, Thompson J, Gardiner DL and Trenholme KR (2008). Sex in *Plasmodium*: a sign of commitment. Trends Parasitol. 24: 168-175.

Graves PM, Carter R and McNeill KM (1984). Gametocyte production in cloned lines of *Plasmodium falciparum*. Am. J. Trop. Med. Hyg. 33: 1045-1050.

Greenwood B (2008). Progress in malaria control in endemic areas. Travel Med. Infect. Dis. 6: 173-176.

LeRoux M, Lakshmanan V and Daily JP (2009). *Plasmodium falciparum* biology: analysis of *in vitro* versus *in vivo* growth conditions. Trends Parasitol. 25: 474-481.

MR4 (2014). The Malaria Research and Reference Reagent Resource Center, American Type Culture Collection, Manassas, VA, USA. Catalog of parasitic protozoa. Retrieved September 3, 2014. Available online at:
https://www.mr4.org/MR4ReagentsSearch/Parasitic%20Protozoa.aspx

Murray CJ, Rosenfeld LC, Lim SS, Andrews KG, Foreman KJ, Haring D, Fullman N, Naghavi M, Lozano R and Lopez AD (2012). Global malaria mortality between 1980 and 2010: a systematic analysis. Lancet 379: 413-431.

Read M and Hyde JE (1993). Simple *in vitro* cultivation of the malaria parasite *Plasmodium falciparum* (erythrocytic stages) suitable for large-scale preparations. Methods Mol. Biol. 21: 43-55.

Saliba KS and Jacobs-Lorena M (2013). Production of *Plasmodium falciparum* gametocytes *in vitro*. Methods Mol. Biol. 923: 17-25.

Taylor-Robinson A.W. and Looker M (1998). Modulatory effect of nitric oxide on *Plasmodium falciparum* male gametocyte exflagellation. In: Moncada S, Toda N, Maeda H, Higgs EA (eds), The Biology of Nitric Oxide, part 6. Portland Press, London, UK. pp. 248-249.
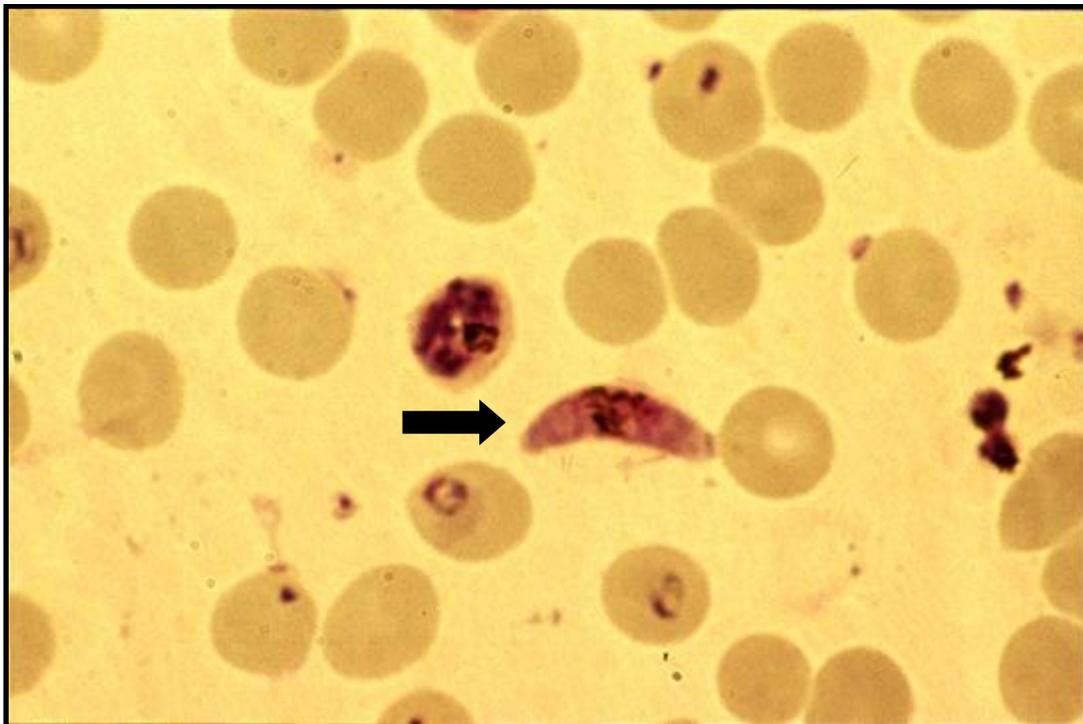
FIGURE LEGENDS

Sexual Stages Of The Malaria Parasite *Plasmodium Falciparum* Cultivated *In Vitro*
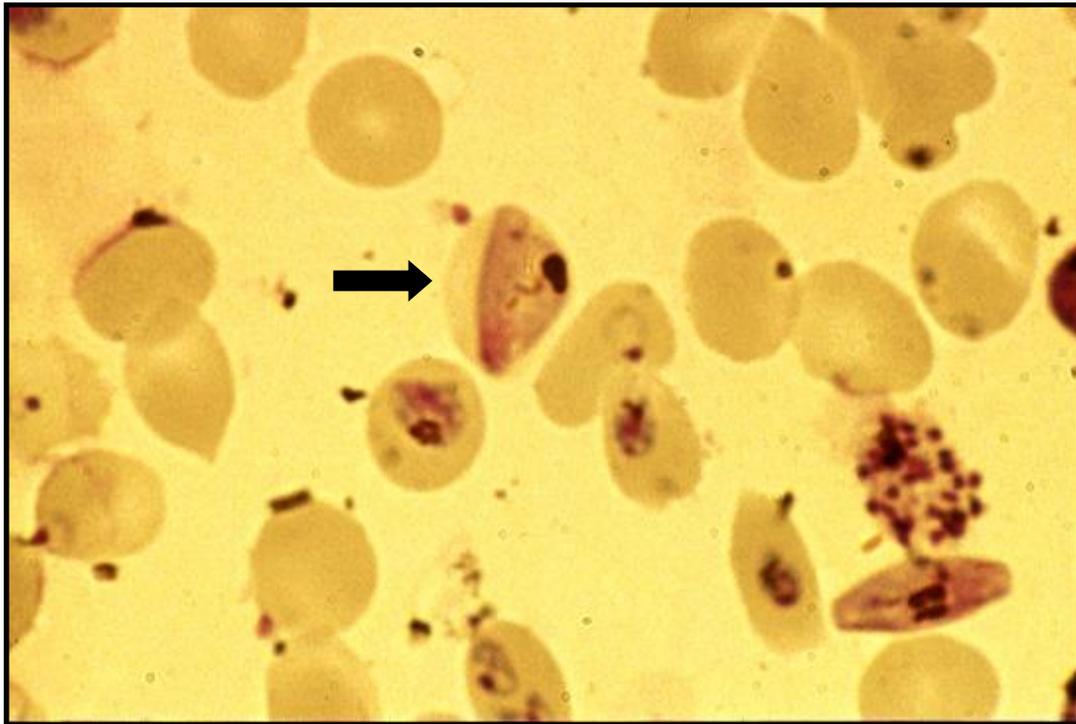

Figure 1. Stage II (central, arrowed) and stage III (bottom right, arrowed) immature gametocytes (blood film, wet mount, x1000 magnification under oil immersion).
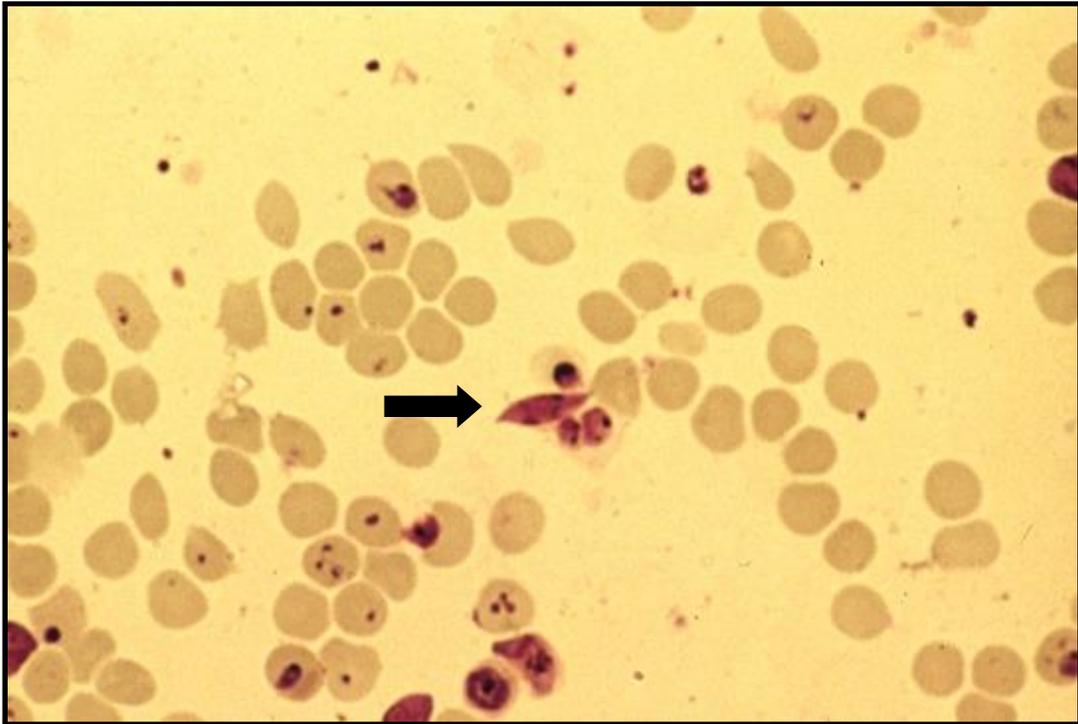
Figure 2. Stage IV immature gametocyte (arrowed) (blood film, wet mount, x400 magnification).

Figure 3. Stage V mature gametocyte (arrowed), showing characteristic sausage-shaped morphology (blood film, wet mount, x1000 magnification under oil immersion).
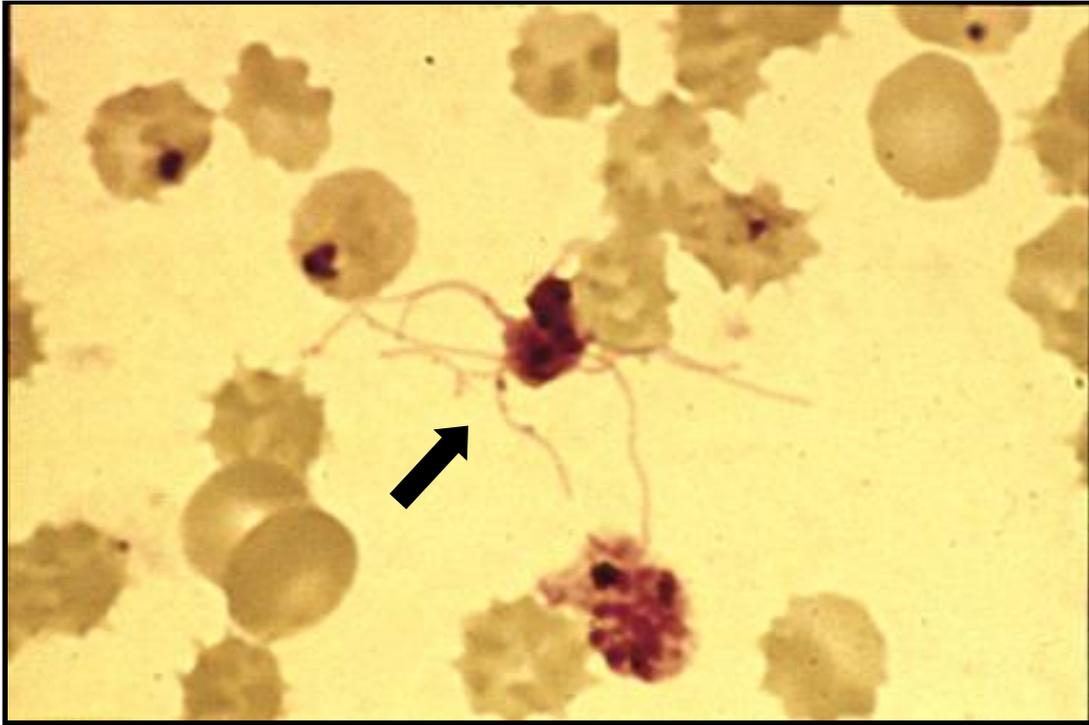
Figure 4. Male (micro)gametocyte exflagellation (arrowed) - extrusion of motile, flagella-like microgametes with vigorous movement (blood film, wet mount, x1000 magnification under oil immersion) (an unusually clear picture of this metabolically dynamic and visually striking event).