

In Vitro Culture of *Plasmodium falciparum*: Obtention of Synchronous Asexual Erythrocytic Stages

María Roncalés*, Jaime Vidal, Pedro A. Torres, Esperanza Herreros

GlaxoSmithKline R&D, Tres Cantos Medicine Development Campus, Malaria Discovery Performance Unit,
Madrid, Spain

Email: maria.p.roncales@gsk.com

Received 23 January 2015; accepted 24 February 2015; published 27 February 2015

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Abstract

Cultivation of the erythrocytic stages of *Plasmodium* parasites, specifically the most important and deadly for humans, *Plasmodium falciparum*, has required a lot of effort and time in order to develop a continuous *in vitro* culture. Moreover, the development of methods to synchronize *P. falciparum* parasites (which grow asynchronously *in vitro*) has become an essential tool in research to study different immunological, biochemical or physiological aspects of the parasite. We have compared two different synchronization methods, one based on differential permeability of the membrane of parasitized erythrocytes, and the other on the sedimentation behavior in gelatin solution. An optimized method has been established which allows for maintaining a healthy, highly synchronous culture for longer periods of time. Asexual erythrocytic stages of a complete *P. falciparum* cycle have been obtained, which is the starting point of the stage-specific assays of the activity of new antimalarial drugs.

Keywords

P. falciparum, Synchronous, Asexual Stages, Stage Specificity

1. Introduction

Malaria is the most important parasitic infection in humans. Globally, 3.2 billion people in 97 countries and territories are at risk of being infected with malaria and developing disease. In 2014, 198 million cases of malaria occurred, causing 584,000 deaths, especially in sub-Saharan African countries, where children under 5 years

*Corresponding author.

were the most affected population group [1].

Malaria is caused by five species of parasites belonging to the genus *Plasmodium*. Four of these (*P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*) are human malaria species, which are transmitted from one person to another by female mosquitoes of the genus *Anopheles* [2]. The life cycle of this organism is complex, with the parasite alternating between sexual reproduction (sporogonic phase) in an invertebrate host (mosquito) and asexual reproduction in a vertebrate host (schizogonic phase, with proliferation of the parasites in erythrocytic and exoerythrocytic sites) [3]. *In vitro* cultivation of malaria parasites requires simulation of the conditions of both phases with the specific characteristics and properties of each of them. The greatest effort and time have been invested in cultivation of the erythrocytic stages of the *Plasmodium* life cycle, specifically in the most important and deadly of the human malarial parasites, *P. falciparum*. Trager and Jensen pioneered the development of a procedure for continuous *in vitro* cultivation of the erythrocytic stages of the human malaria parasite [4].

In human host, *P. falciparum* exhibits a synchronous life cycle for approximately 48 hours. A blood sample taken at any time from an infected host will show a parasite population at the same stage of the developmental cycle, e.g. mostly ring stages or schizonts [3]. However, natural synchronization of parasites in man (due to *P. falciparum*) is lost in culture, making more difficult to collect specific developmental stages of the parasite during *in vitro* growth [4]-[6]. Synchronization of *P. falciparum* has therefore become an essential tool to characterize its specific stages: studying the changes that occur in the parasitized erythrocytes, identifying stage-specific proteins, glycoproteins and antigens, etc. A better knowledge of *P. falciparum* parasites may improve the development of new drugs and vaccines against malaria.

Various synchronization methods have been reported considering different properties such as temperature cycling [7], magnetic separation [8], and physical separation based on differential density [9], osmotic lysis and permeability properties [3] [6]. The most common and well established method to synchronize the intraerythrocytic stages of *P. falciparum* is sorbitol treatment, because it is versatile and simple to perform [6] [10]. An alternative method for *Plasmodium* culture synchronization that allows for concentration of the older parasite stages is the one based on sedimentation behavior in gelatin solution (Plasmion) [11] [12].

This study compares the synchronization method described by Lambros and Vanderberg based on sorbitol treatment [6] to the method reported by Ter Kuile, which combines sorbitol treatment with the synchronization method based on differential density (Plasmion) [11] [12]. Some modifications have been considered, such as sequential sorbitol treatment of parasite cultures each 48 hours to improve the degree of culture synchrony and viability. As a result, an optimized synchronization method has been established that maintains a high degree of synchrony of the parasites and optimal culture conditions for long periods of time. The “0-hour start time” for a synchronous complete cycle of 48 hours of the erythrocytic stages has also been defined. This new method allows for isolation of all erythrocytic asexual stages: early rings, late rings, early trophozoites, late trophozoites, early schizonts, and late schizonts, with the estimated time of appearance of each stage well defined, to study the stage-specific activity of new antimalarial drugs.

2. Material and Methods

2.1. Culture Medium

RPMI 1640 with 25 mM HEPES, sodium bicarbonate and glutamine (GIBCO™ ref: 52400-025), supplemented with 10% of pooled human sera AB (14-490 E Cambrex) and 0.15 mM of hypoxanthine (from HT supplement ×50, GIBCO™ ref: 41065). Human sera are decontaminated 30 minutes at 56°C, aliquoted and stored frozen at -20°C until use. Complete medium is usually prepared fresh just before use and pre-warmed at 37°C.

2.2. Red Blood Cells (RBC)

Red blood cells stock suspensions are prepared from whole blood bags coming from incomplete blood donation, provided by the Spanish Red Cross (<25 days after sampling). Whole blood is aliquoted and stored at 4°C. To prepare red blood cells, whole blood is centrifuged and washed 3 times with serum-free RPMI by centrifugation (10 minutes at 2000 rpm). The upper phase, containing white blood cells, is removed. Washed red blood cells are kept as a 50% suspension in complete medium, and stored for a maximum of 4 days at 4°C. The human biological samples were sourced ethically, and were used for research in compliance with the terms of the informed consents.

2.3. Parasites

P. falciparum 3D7 strain was obtained from the MR4 Resource Center (ATCC) and was maintained in complete medium at a hematocrit of 5% in continuous culture using a method adapted from Trager and Jensen [4]. Parasitemia was calculated by counting the percentage of parasitized erythrocytes by light microscopy (at least 5000 RBCs). Growth factor was calculated by dividing final parasitemia into initial parasitemia. Thin blood films were made every day from each culture flask, fixed with methanol, and stained for 10 minutes in Giemsa (Merck, ref: 1.09204) at 5% in buffered water pH 7.2 (Buffer tablets, Merck). The different stages of development (early and late rings, early and late trophozoites, and early and late schizonts) were determined and counted according to Silamunt *et al.* [13]. Culture was maintained in 75 cm² culture flasks (canted neck, Corning) at 37°C, under low oxygen atmosphere (5% CO₂, 5% O₂, 95% N₂).

2.3.1. Synchronization of Parasite Culture

To establish synchrony, two different methods were considered: **Sorbitol treatment** (isolation of ring stage) and **Plasmion treatment** (isolation of trophozoite and schizont stage). Sorbitol solution: 5% (w/v) of sorbitol (Sigma S 6021) in cell culture grade water (Sigma W3500). Plasmion solution (Laboratoire Fresenius Kabi, France).

Sorbitol treatment to synchronize cultures: This synchronization method, described by Lambros and Vanderberg [6], is based on the differential permeability of parasitized RBC membrane. While RBCs are naturally impermeable to sorbitol, infected RBCs with mature stages have a permeable membrane due to the structural modifications induced by the parasite. This property is used to selectively kill mature forms of the parasite by osmotic shock without affecting uninfected RBCs and RBCs parasitized by ring stages. Pre-warm an aliquot of 5% D-sorbitol, complete medium, and RPMI at 37°C. Centrifuge the culture at 1800 rpm for 5 minutes at room temperature, discard the supernatant, and resuspend in 5 volumes of pellet of pre-warmed sorbitol solution. Homogenate with the pipette and incubate for 5 minutes at 37°C. Centrifuge 5 minutes at 1800 rpm. Remove the supernatant, and wash twice with 25 mL of complete medium. After the last wash, remove the supernatant and adjust the pellet to a hematocrit of 50% and use it to inoculate a culture in a 75 cm² culture flask, adding fresh RBCs to maintain hematocrit at 5% for synchronous cultures.

Plasmion treatment to synchronize cultures: A method based on sedimentation behavior in gelatin solution which also selects the phenotype of knob+ parasites. In a Plasmion solution, the knob+ stages float, while the younger stages (rings) sediment like uninfected RBCs. This property is used to select mature parasites stages (trophozoites and schizonts). Pre-warm an aliquot of Plasmion. Remove most old medium from the flask culture and transfer to a 15 mL tube. Centrifuge 5 minutes at 18000 rpm at room temperature, discard culture medium and read the pellet volume. Add 1.4 mL of complete medium and 2.4 mL of Plasmion solution for each mL of cell pellet and mix gently. After incubating for 30 minutes at 37°C without any movement to avoid disturbing sedimentation, take the supernatant (containing the older stages of the parasite, trophozoites and schizonts) and use it to make a dilution or to establish the specific culture conditions needed.

2.3.2. Sorbitol 34 Hours

Method described by Lambros and Vanderberg (1979) with some modifications [6]. Two sequential treatments with sorbitol were applied each 48 hours. Thirty-four hours after the second treatment, a third sorbitol treatment was used to obtain young rings. After the first sorbitol treatment, the culture (75 cm² culture flasks, 20 mL total volume) was adjusted to 1% parasitemia and 5% hematocrit. A medium change was made between the two treatments (24 hours of culture). Percentage of parasitemia and predominant stage were evaluated by Giemsa staining every 24 hours. From 72 hours of culture, the culture was evaluated every two hours (total culture time, 96 hours). A Giemsa smear was also made after each sorbitol treatment in order to measure the effect of the method. This synchronization method was made twice with three replicates each time.

2.3.3. Sorbitol 6 Hours

Method described by Ter Kuile *et al.* (1993) with some modifications [11]. Two sequential treatments with sorbitol were applied each 48 hours. Twenty-four hours after the second sorbitol treatment, a Plasmion treatment was made. Six hours after this Plasmion treatment, a third sorbitol treatment was used. After the first sorbitol treatment, the culture (75 cm² culture flasks, 20 mL total volume) was adjusted to 1% parasitemia and 5% hematocrit. A medium change was made between the two sequential sorbitol treatments (24 hours of culture).

Percentage of parasitemia and predominant stage were evaluated by Giemsa staining every 24 hours. After 2 and 6 hours of Plasmion treatment, the culture was also evaluated (total time of culture: 168 hours). A Giemsa smear was also made after each synchronization treatment in order to measure the effect of the specific method. This synchronization method was made twice with three replicates each time.

3. Results and Discussion

Different sorbitol and plasmion cycles were used to establish an optimized synchronous method. Two different synchronization methods, one based on differential permeability (Sorbitol 34 hours) and the other on sedimentation behavior (Sorbitol 6 hours), were compared.

3.1. Sorbitol 34 Hours

This synchronization method started with a sorbitol treatment after which the culture was adjusted to 1% parasitemia and 5% hematocrit (Figure 1). Due to sorbitol treatment, the culture at this initial time is enriched in ring stage (80%). Twenty-four hours later, the percentage of parasitemia is almost the same (1.15%), but trophozoites predominate due to culture maturation. Once the first cycle is completed (48 hours of culture), alternation of the stage has been produced (97% of new ring stages obtained after reinvasion of the mature forms) with a high growth factor (5.87), reaching a percent parasitemia of 6.75. A second sorbitol treatment was made at this time. High parasitemia with a high percentage of rings is required as an initial condition for each synchronous sorbitol treatment to achieve a healthy culture, all the more so if it will be used for sequential synchronization methods. At 72 hours of incubation, trophozoites predominated, and to determine the schizonts peak, culture samples were taken at frequent intervals (every 2 hours) during this transition period. In agreement with Lambros and

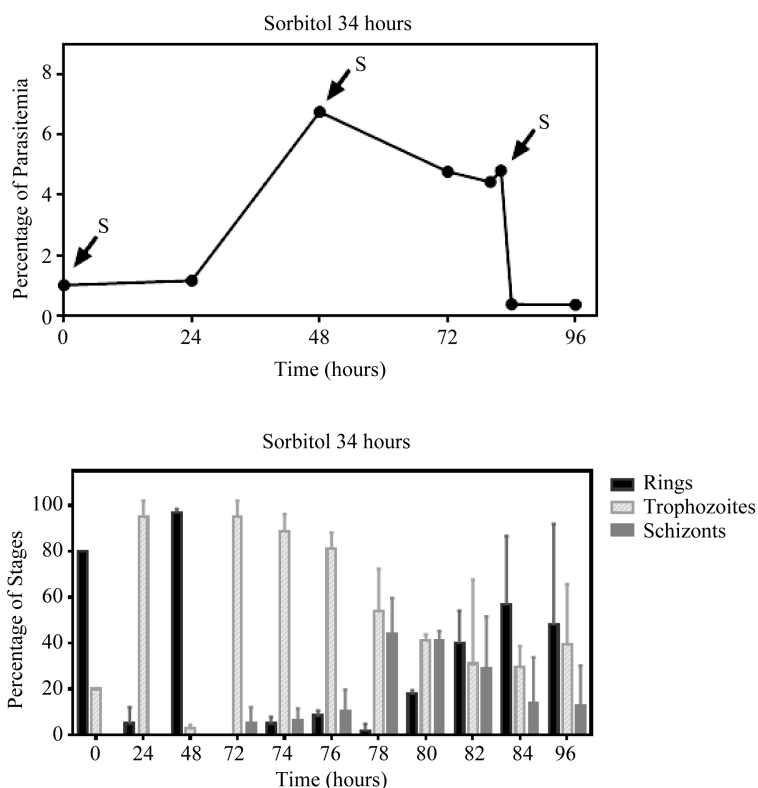


Figure 1. Percentage of parasitemia during the total culture time (96 hours) is represented in the top panel. Arrows indicate the specific synchronization treatment applied (S: Sorbitol). The bottom panel shows the percentage of each stage (Black: ring stages; Strip-Grey pattern: trophozoite stages; Dark grey: schizont stages).

Vanderberg results [6], the schizont peak was achieved at 78 - 80 hours of culture. Thereafter, as the schizont population decreased, a concomitant increase occurred in the frequency of ring forms due to invasion of uninfected RBCs by merozoites coming from merogony and schizont lysis. A third sorbitol treatment was applied 34 hours after the second one to obtain a greater degree of synchrony. At this time (82 hours of culture), the proportion of rings (40%) was not high enough to maintain a high parasitemia in the synchronous culture due to the reduction in parasitemia observed after the third sorbitol treatment (93%). In our hands, three sorbitol treatments in less than two complete cycles of the parasites affected parasite viability. Gametocytes were observed at 96 hours, which is a clear signal of culture stress [14] (Figure 1).

3.2. Sorbitol 6 Hours

This synchronization method was based on the properties of the Plasmion solution (that allows for purification of mature parasites without affecting their viability) combined with three sequential sorbitol treatments (two each 48 hours of culture and the third one 6 hours after Plasmion treatment). Results of this method are illustrated in Figure 2. After the first sorbitol treatment, culture was adjusted to 1% parasitemia and 5% hematocrit. At this initial time, percentage of rings was maximum (100%). Twenty-four hours later, percentage of parasitemia was almost the same (1.34%) but trophozoites predominated due to culture maturation. Once the first cycle was completed (48 hours of culture), alternation of the stage occurred (99% of new ring stages obtained after reinvasion of the mature stages) with a growth factor of 4.83, reaching a percentage of parasitemia of 6.47. A second sorbitol treatment was used at this time. This high parasitemia with a high proportion of rings (99%) is essential for culture survival. At 72 hours of culture, trophozoites predominate (99%), maintaining percentage of parasitemia (6.89%). At this time, a Plasmion method was performed, adjusting the percentage of parasitemia after treatment to 1.3% - 1.5%. As described by Ter Kuile [11], a third sorbitol treatment was made 6 hours after

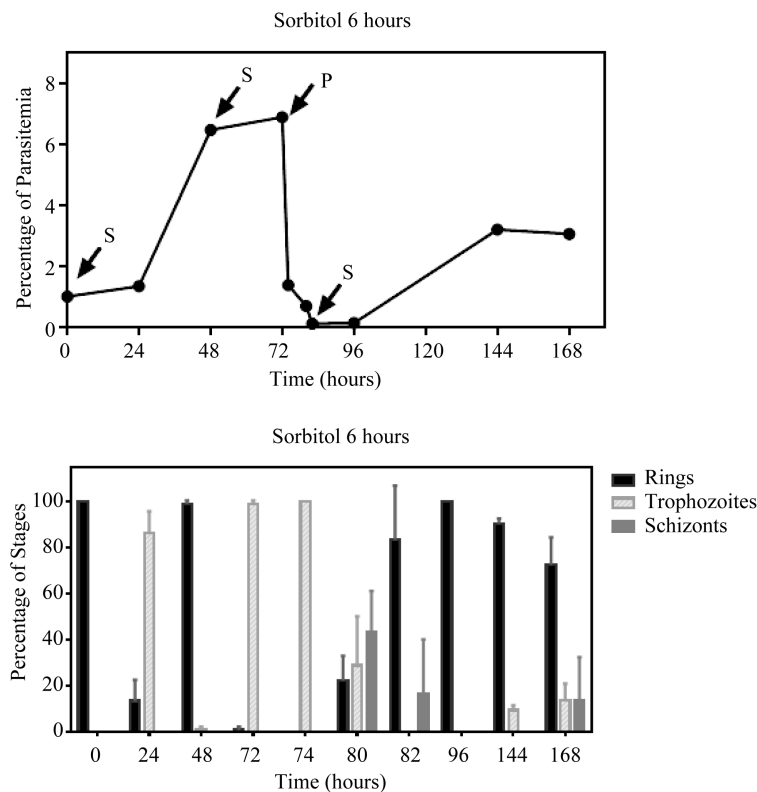


Figure 2. Percentage of parasitemia during the total culture time (168 hours) is represented in the top panel. Arrows indicate the specific synchronization treatment applied (S: Sorbitol; P: Plasmion). The bottom panel shows the percentage of each stage (Black: ring stages; Strip-Grey pattern: trophozoite stages; Dark grey: schizont stages).

Plasmion treatment. The ring stage was significantly enriched after sorbitol (83%), but percentage of parasitemia was not so high (0.11%) due to the initially low proportion of rings at this moment (24%). Parasites grow until 168 hours of culture, although the predominant stage at this time was the opposite as expected (rings instead of trophozoites). Parasitemia also decreased (from 3.20 to 3.06; no gametocytes observed), showing that the culture was not so healthy due to the dramatic decrease in parasitemia occurring after the third sorbitol treatment (0.11%).

These synchronizations methods (sorbitol and Plasmion) are essential tools to study different aspects of malarial parasites, such as immunological, biochemical, and physiological differences between the different stages of parasite development. In order to obtain healthy parasites to guarantee their survival during such studies, some factors have been considered. Based on our results, sorbitol treatment is a more aggressive method than Plasmion treatment: gametocytes stages were observed during sequential sorbitol protocols (more than two sorbitol treatments in less than 48 hours), while no gametocytes were observed during Plasmion treatment. For sorbitol treatment, a very important limiting factor is the proportion of rings at the start of the process. For example, a 29% of parasitemia reduction (from 6.75% before sorbitol to 4.77% after sorbitol) was observed, when the proportion of rings at the initial time was 99%. By contrast, if the proportion of rings was 40%, parasitemia reduction increased to 93% (from 4.80% before sorbitol to 0.36% after sorbitol). Ring age may also influence development of the treatment: late rings (16 - 26 hours) are more susceptible to sorbitol (due to permeability changes in these erythrocytes parasitized by rings), so that at this point, age of the rings may have influenced sorbitol efficiency. Treatment with Plasmion allows for achieving a higher concentration of mature stages (trophozoites and schizonts) very easily and quickly without affecting parasite viability. An important factor to consider is the reinvasion capacity of the mature stages, which may be compromised at high parasitemia levels.

Two different assays were performed to establish the best conditions for reinvasion (with rings or trophozoites at initial time of culture) (**Figure 3**). Parasites with ring stages (99%) were adjusted to 1% or 0.5% parasitemia and 5% hematocrit (asynchronous culture) to compare them to a culture adjusted to the same initial conditions but with a previous sorbitol treatment (**Figure 3(a)**). These parasites were cultured for 48 hours, after which higher parasitemias levels were reached in the cultures treated with sorbitol (final parasitemia levels of 9.45% for cultures started at 1% and 6.59% for cultures started at 0.5%). An additional assay was done to check if the mature stages could compromise reinvasion and survival of the cultures (**Figure 3(b)**). Cultures enriched in mature stages (>80%) were cultured for 72 hours in order to assess whether different initial parasitemia levels (2% or 0.5%) could influence the final parasitemia obtained. The highest parasitemia (14.78%) was achieved with the culture started at 0.5%. During the first reinvasion of the mature stage, growth factors were similar (3.7 for cultures started at 2% and 4.34 for cultures started at 0.5%). Greater differences were observed during the second reinvasion (48 to 72 hours), where the growth factor for cultures started at 2% was 1.86, while the growth factor for cultures started at 0.5% increased to 7.8. As culture time progresses, the reinvasion capacity of the mature stages may be compromised by increasing parasitemia.

Considering all these conditions (percentage of predominant stages before synchronization treatment, initial

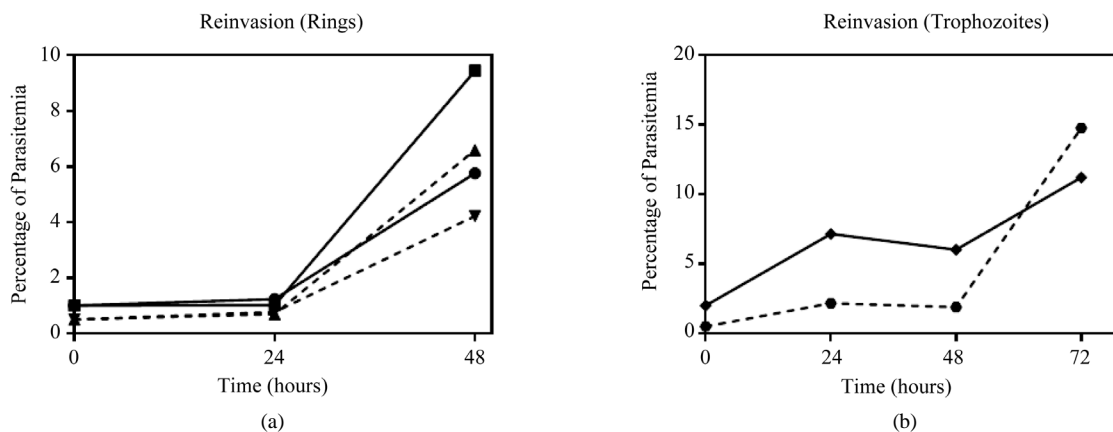


Figure 3. Reinvasion conditions of different parasite stages (a)—Ring stages; (b)—Trophozoite stages. (a): synchronous culture at 1% (■), asynchronous culture at 1% (●); synchronous culture at 0.5% (▲) and asynchronous culture at 0.5% (▼). (b): dark line—cultures started at 2% parasitemia, dotted line—cultures started at 0.5% parasitemia.

parasitemia, growth factor, etc.) a method for optimal synchronization of cultures was established (**Table 1**).

This method combines two sequential sorbitol treatments (each 48 hours) and a Plasmion treatment at 24 hours of culture (**Figure 4**). Good culture synchronization is maintained throughout culture duration, growing with an increase in parasitemia of 4-fold to 9-fold after each Plasmion treatment (increasing the ratio as well as the culture is synchronized during more time) and showing a good reinvasion capacity of the mature stages; parasite viability is also well preserved (no gametocytes observed). The overall increase in parasitemia after

Table 1. Synchronization method established for 3D7 *P. falciparum* parasites.

Time (h)	CULTURE	TREATMENT	OBSERVATIONS
0	Asynchronous	Sorbitol	At least 1.5% parasitemia after treatment Consider decrease in parasitemia (80%)
	5% - 7%p		
	90% rings		
24	5% Hematocrit	Plasmion	Consider mature stage reinvasion ($\times 4$ up to $\times 9$ when synchrony increases)
	Mature stages		
48	Ring stages	Sorbitol	Consider age of rings (Rings of 0 - 16 h, best results)
72	Mature stages	If %p > 2: Plasmion	High parasitemia in mature stages may compromise viability
		If %p < 2: dilution to adjust to 1% parasitemia	
96	Rings stages	If %p > 3 dilution: to adjust to 3% parasitemia	The next day will be the same %parasitemia with mature stages
120	Mature stages	Medium change/Plasmion	Depending on degree of synchrony: Plasmion if % mature stages < 90% Medium change if synchrony is high
144	Ring stages	Sorbitol/Dilution	Sorbitol if there are still mature stages Dilution to keep the culture until assay

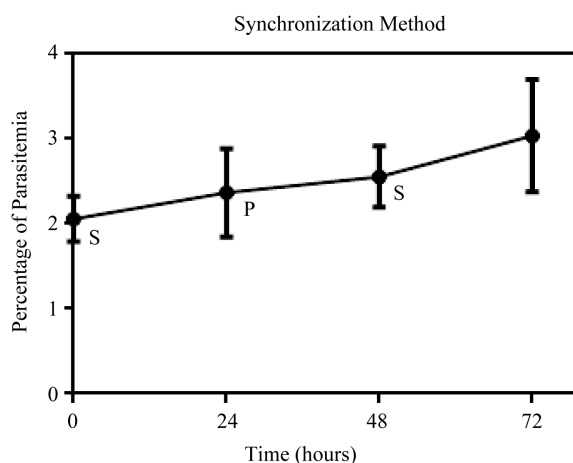


Figure 4. Percentage of parasitemia (mean of three replicates) of cultures started at 2% parasitemia and 5% hematocrit with two sequential sorbitol treatments (at 0 and 48 hours of culture) and a Plasmion treatment at 24 hours of culture (S: Sorbitol; P: Plasmion).

Plasmion treatment is the same as reported by other authors [5] [12].

This method may be used to synchronize cultures for months, thus ensuring survival and a high degree of synchrony of parasites. As compared to other methods described using low hematocrit conditions [15], which are labor-intensive (requiring culture medium changes at any time for at least two weeks), our optimized method is simple to develop, maintains culture synchrony, and avoids stress to the parasites. This procedure (Sorbitol-Plasmion-Sorbitol) may be repeated every week taking into account the observations in **Table 1**, achieving a highly synchronous culture. To determine the specific age of each asexual erythrocytic stage in a complete cycle of 48 hours, it would be advised to perform a treatment with Plasmion (as a last synchronization step). Change over time after Plasmion treatment is illustrated in **Figure 5**.

Ring stage was observed 6 hours after Plasmion treatment (57%), and the highest percentage of rings (95%) was found 10 hours after Plasmion treatment. The “0-hour start time”, for a synchronous complete cycle of 48 hours of the erythrocytic stages has been defined at this point. It is very important to consider the maturity of trophozoite stages and, even more, the presence of early schizonts (two or three nuclei) when Plasmion treatment is applied, because the greater degree of maturity of trophozoites and even more schizonts may influence determination of the “0-hour start time” (95% of early rings obtained), which will be earlier when these stages (trophozoites and schizonts) are very mature. Another advantage of Plasmion treatment is that early and late schizonts are obtained without the need to combine a percoll gradient with sorbitol treatment [15]. Some authors [10] [15] recently reported a new synchronization method based on the combination of sorbitol treatment with percoll gradients and magnetic column purification to achieve high parasitemia levels (up to 40%). These methods are however time-consuming and sometimes lead to loss of synchrony.

In agreement with other authors [11], [13] and [16], isolation of all erythrocytic asexual stages—early rings, late rings, early trophozoites, late trophozoites, early schizonts and late schizonts—is now available, and their specific time of production is defined as follows: early rings at 0 - 7 hours, late rings at 15 - 22 hours, early trophozoites at 20 - 27 hours, late trophozoites at 25 - 32 hours, early schizonts at 30 - 37 hours, and late schizonts at 40 - 45 hours (**Figure 6(b)**). The synchronous culture develops healthily throughout the 48 hours of the asexual cycle, reaching a final parasitemia of 7.64% at the end of the cycle (100% new rings), maintaining synchrony throughout the cycle (**Figure 6(a)**).

The new synchronization method has been well characterized and allows for obtaining the specific asexual erythrocytic stages of a complete 48-hours cycle of *P. falciparum* parasites. In most studies, the establishment of highly synchronized cultures of *Plasmodium falciparum* has become essential. These being isolation of early rings, late rings, early trophozoites, late trophozoites, early schizonts and late schizonts [13] and [16], for example, to study the changes in the structure of parasitized red blood cells for immunological, biochemical and physiological studies [17], [6], or to study the specific stage more susceptible to new antimalarials [13]. In fact, this optimized method has been used as a procedure to obtain the different stages of the asexual erythrocytic cycle, and to test novel compounds candidate to become antimalarial drugs.

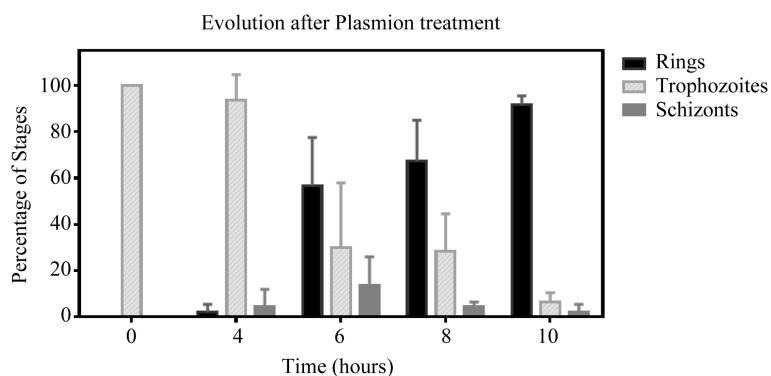


Figure 5. Change over time in different parasite stages after Plasmion treatment. Parasites were adjusted to 0.5% parasitemia and 5% hematocrit after Plasmion treatment, and changes over time in stages were measured every two hours (mean of three replicates). (Black: ring stages; Strip-Grey pattern: trophozoite stages; Dark grey: schizont stages).

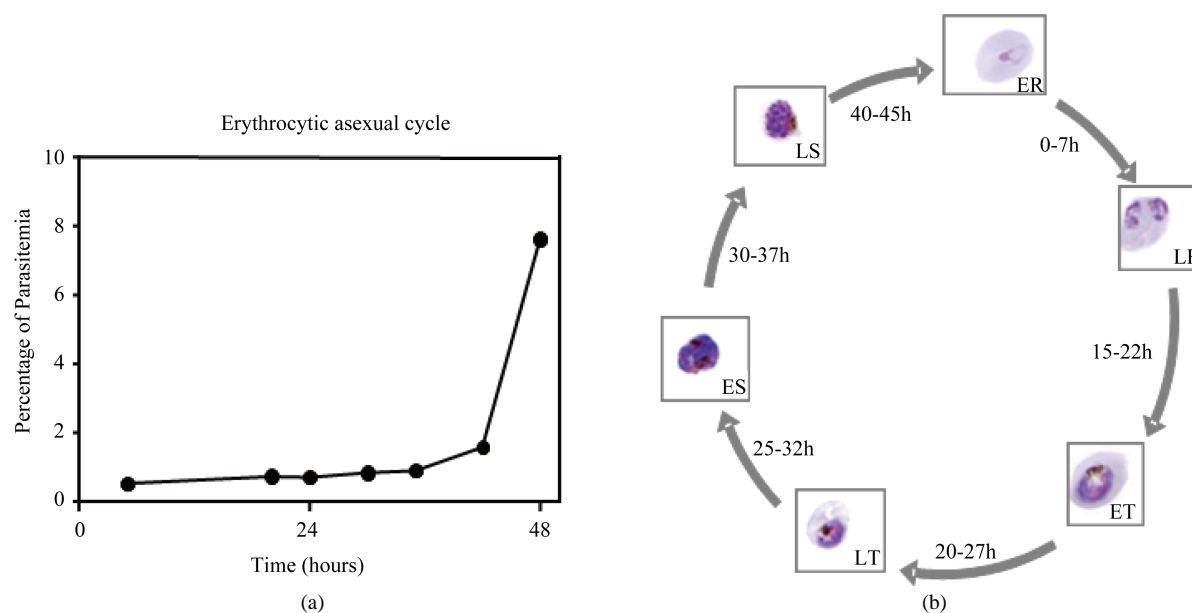


Figure 6. Complete erythrocytic 48-hours asexual cycle of highly synchronous *P. falciparum* parasites. ((a): Change over time in the synchronous culture during 48 hours (mean values of three cultures). Initial conditions: 0.5% parasitemia and 5% hematocrit). ((b): Age culture of the different stages of *Plasmodium falciparum* highly synchronized culture. (ER: early ring; LT: late ring; ET: early trophozoite; LT: late trophozoite; ES: early schizont; LT: late schizont)).

4. Conclusion

Standard synchronization methods of *P. falciparum* cultures (sorbitol and Plasmion) were compared to assess the advantages of each of them (obtention of early stages (rings) with sorbitol treatment and late stages (trophozoites and schizonts) with Plasmion treatment). An optimized method was established that maintains the high degree of synchrony of the parasites and optimal culture conditions for long periods of time. The specific asexual erythrocytic stages of a complete 48-hour cycle of *P. falciparum* parasites have been defined, and their specific time of emergence has been determined, in order to be used for stage specificity assays to characterize the stage-specific activity and rates of action of novel antimalarial drugs.

Acknowledgements

The authors are indebted to our partners at the Medicines for Malaria Venture, who not only helped to support this work financially but also provided advice and facilitated collaborations within the malaria scientific community.

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