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Refrigeration provides a simple means to synchronize *in vitro* cultures of *Plasmodium falciparum*

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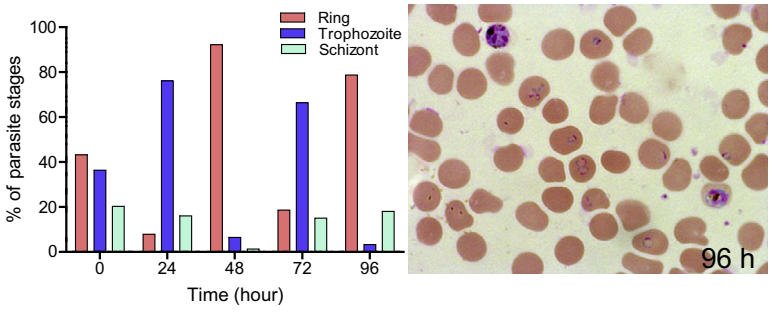
HIGHLIGHTS

- *P. falciparum in vitro* culture can be synchronized by storage at 4 °C for 8–24 h.
- This simple refrigeration method can achieve >70% synchrony.
- Synchronized parasites can be used for downstream work such as *in vitro* drug assay.

GRAPHICAL ABSTRACT

P. falciparum culture can be synchronized by storage at 4 °C and the synchrony could remain for at least two intraerythrocytic cycles.



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ABSTRACT

Plasmodium falciparum is usually asynchronous during *in vitro* culture. Highly synchronized cultures of *P. falciparum* are routinely used in malaria research. Here, we describe a simple synchronization procedure for *P. falciparum* asexual erythrocytic culture, which involves storage at 4 °C for 8–24 h followed by routine culture. When cultures with 27–60% of ring stage were synchronized using this procedure, 70–93% ring stages were obtained after 48 h of culture and relative growth synchrony remained for at least two erythrocytic cycles. To test the suitability of this procedure for subsequent work, drug sensitivity assays were performed using four laboratory strains and four freshly adapted clinical *P. falciparum* isolates. Parasites synchronized by sorbitol treatment or refrigeration showed similar dose–response curves and comparable IC_{50} values to four antimalarial drugs. The refrigeration synchronization method

Abbreviations: DHA, dihydroartemisinin; PPQ, piperaquine; CQ, chloroquine; QN, quinine.

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Refrigeration
Drug susceptibility

is simple, inexpensive, time-saving, and should be especially useful when large numbers of *P. falciparum* culture are handled.

1. Introduction

Malaria continues to be a leading cause of morbidity and mortality worldwide. It is responsible for more than 250 million cases annually, resulting in almost one million deaths. Four *Plasmodium* species (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale*) cause human malaria and the monkey parasite *Plasmodium knowlesi* has emerged recently as a threat to cause zoonotic malaria in human populations of Southeast Asia (Collins, 2012). Malaria parasite development in human hosts is highly synchronous. Synchronous rupture of intraerythrocytic schizonts and the appearance of their progeny in the circulating blood are associated with the most unique clinical feature of malaria – paroxysmal fever.

The development of *in vitro* culture technique of the human malaria parasite *P. falciparum* has enabled studies of many biological aspects of this parasite (Trager and Jensen, 1976). During *in vitro* culture, *P. falciparum* development is usually asynchronous with all asexual stages of the parasite present at any given time. The generation of cultures containing highly synchronized parasites is necessary for various studies. Various synchronization methods have been established, which rely on removal of different parasite stages by differential osmotic lysis or chemical poison (Lambros and Vanderberg, 1979; Pfaller et al., 1982), physical separation relying on differential density (Aley et al., 1984; Jensen, 1978; Mrema et al., 1982), magnetic separation (Ahn et al., 2008; Bhakdi et al., 2010; Heidrich et al., 1982; Paul et al., 1981), or cell cycle or DNA polymerase inhibitors (Assaraf et al., 1986; Heidrich et al., 1982; Hensmann and Kwiatkowski, 2001; Hoppe et al., 1991; Hui et al., 1983; Inselburg and Banyal, 1984; Lelievre et al., 2005; Naughton and Bell, 2007; Scragg et al., 1999). Sometimes, two of the these methods are combined to achieve higher levels of synchronization and a narrow window of parasite development (Inselburg, 1983; Jensen, 1978; Lelievre et al., 2005; Pasvol et al., 1978; Radfar et al., 2009; Ranford-Cartwright et al., 2010; Spadafora et al., 2011). Temperature cycling has also been reported as a means to improve synchronization (Haynes and Moch, 2002). This method achieves synchronization by cycling the temperature between 17 and 40 °C in several complicated steps.

Here we report a novel physical synchronization method of *P. falciparum in vitro* culture based on differential sensitivity of different asexual erythrocytic stages to refrigeration, which can yield 70–93% ring stage parasites. The method is simple to perform, requires no specialized equipments or reagents, and is suitable for *in vitro* drug sensitivity assay.

2. Materials and methods

2.1. Parasites and in vitro culture

P. falciparum laboratory strains 3D7, HB3, DD2, and 7G8 obtained from the Research and Reference Reagent Resource Center (MR4) (Manassas, VA) and four clinical isolates WB299, WB548, WB183 and WB682 collected from the China–Myanmar border area were cultured and used for *in vitro* drug assay. Parasites were routinely cultured in fresh type O human red blood cells (RBCs) at a 5% hematocrit in a complete medium [RPMI 1640 (10.4 g/liter), HEPES (5.94 g/L), hypoxanthine (50 mg/L), Albumax II (5 g/L), gentamicin (50 mg/L), and $NaHCO_3$ (2.1 g/L)] at 37 °C in 25 cm^2 flasks (Costar) under a gas environment of 92% N_2, 5% CO_2, and 3% O_2. Synchronization of parasite cultures with 5% sorbitol (Sigma) was performed as previously described (Lambros and Vanderberg, 1979).

2.2. Parasite synchronization by refrigeration

To determine the effect of temperature and treatment time on parasite development, cultures of the *P. falciparum* 3D7 clone at 40–50% of ring stage were stored at either 4 or 8 °C for varying intervals (4, 6, 8, 10, and 24 h). After the refrigeration treatment, cultures were removed and continuously cultured at 37 °C for 48 h. These cultures were examined at 24, 48, 72 and 96 h by microscopy using Giemsa-stained smears to determine parasitemia and proportion of different developmental stages (rings, trophozoites, and schizonts). Using defined cooling conditions (4 °C for 24 h), we further tested whether this method is suitable for synchronizing cultures at different proportions of ring-stage parasites (27–44% and 45–60%). For each treatment, 2–3 biological replications were tested. In subsequent experiments, cultures with >30% ring stage parasite were synchronized by refrigeration at 4 °C for ~24 h.

2.3. The effect of refrigeration on schizont-stage parasites

To purify schizont-stage parasites, synchronized 3D7 parasite culture at schizont stage were loaded on a 40–70% Percoll step gradient. After centrifugation, schizonts were collected from the 40/70 interface. Purified schizonts were diluted to 0.5% parasitemia with fresh RBCs, and divided into two flasks. One flask was stored under 4 °C for 20 h followed by culturing at 37 °C for 36 h. The other flask was used as control and cultured at 37 °C for 36 h without being subjected to the refrigeration treatment. Smears were made from these cultures, stained with Giemsa, and examined by microscopy.

2.4. Comparison of refrigeration and sorbitol treatment methods for synchronization

To compare the synchronization efficiency of the refrigeration and sorbitol treatment methods, cultures of 3D7 parasites were divided into two aliquots. One aliquot was subject to 5% sorbitol treatment, whereas the other was stored at 4 °C for 24 h. Both treatments were followed by culturing under standard conditions for 48 h. Parasite stages were differentiated and counted in Giemsa-stained thin smears. A total of five cultures with different proportions (30–50%) of initial rings were compared between the two synchronization methods.

2.5. Drug assay

To determine the suitability of the refrigeration synchronization method for subsequent drug assays, parasites from four laboratory strains and four field isolates were assayed in parallel for sensitivity to dihydroartemisinin (DHA) (Kunming Pharmaceutical Co., Kunming, Yunnan, China), piperaquine (PPQ) (Chongqing Kangle Pharmaceutical Co. Ltd.), chloroquine (CQ), and quinine (QN) (Sigma). The stock solutions of CQ (3.0 mM) and PPQ (0.3 mM) were