A New Method for Culturing \textit{Plasmodium falciparum} Shows Replication at the Highest Erythrocyte Densities

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\textit{Plasmodium falciparum} replicates poorly in erythrocyte densities greater than a hematocrit of 20%. A new method to culture the major malaria parasite was developed by using a hollow fiber bioreactor that preserves healthy erythrocytes at hematocrit up to 100%. \textit{P. falciparum} replicated equally well at all densities studied. This method proved advantageous for large-scale preparation of parasitized erythrocytes (and potentially immunogens thereof), because high yields (\sim 10^9 in 4 days) could be prepared with less cost and labor. Concomitantly, secreted proteins were concentrated by molecular sieving during culture, perhaps contributing to the parasitemic limit of 8\%–12\% with the 3D7 strain. The finding that \textit{P. falciparum} can replicate at packed erythrocyte densities suggests that this system may be useful for study of the pathogenesis of fatal cerebral malaria, of which one feature is densely packed blood cells in brain microvasculature.

Cerebral malaria, the clinical manifestation of \textit{Plasmodium falciparum} infection, is marked by changes in mental status and coma, with mortality up to 50\%. The causes of cerebral malaria are complex and not completely understood [1]. One prominent pathologic feature is the sequestration of parasitized and normal erythrocytes in cerebral blood vessels [2] at virtually packed densities [3]. It is not known whether \textit{Plasmodium} species can replicate at such high densities. Evidence from the methods of Trager and Jensen [3] for the erythrocytic stage culture of \textit{P. falciparum} does not support replication at high density because replication suffers beyond a hematocrit range of 2\%–20\%.

Here we describe a new method for culture of malaria parasites at high cell density by using a hollow-fiber capillary bioreactor (HFB). This device was first used for cell culture by Knazek et al. [4] and has been widely used for cell expansion and preparative accumulation of cell-derived products such as antibodies [5], cytokines [6], and tumor-associated antigens [7]. In this new culture method, a wide range of hematocrit (up to 100\%) was tested.

\textbf{Materials and methods.} \textit{P. falciparum} strain 3D7 (American Type Culture Collection) was grown in group A–positive erythrocytes in RPMI 1640 medium (Gibco) supplemented with 25 mM HEPES (Gibco), 4.5 mg/mL glucose (Sigma), 0.1 mM hypoxanthine (Gibco), 25 \mu g/mL gentamicin (Gibco), and 0.5\% AlbuMax I (Gibco) according to the method of Trager and Jensen [3]. To assess parasite growth, the cell suspension was stained with acridine orange (Molecular Probes) and the number of parasitized cells in 3000–5000 total counted cells was assessed by fluorescence microscope.

For the \textit{P. falciparum} culture in HFB, we used a medium-size (17.5-mL capacity) apparatus (FiberCell Systems). The system consists of 3 parts: a hollow-fiber cartridge, a pump, and the tubing system that connects the cartridge with a reservoir for medium. Cells are located inside the cartridge between multiple hollow semipermeable fibers with different molecular weight cutoff levels (MWCO; 20 kDa, 5 kDa, or 0.1-\mu m pores) where medium is circulated. The fiber surface area (2100 cm\(^2\) for a medium-size HFB) provides very efficient nutrition, metabolite, and gas exchange between the cell suspension and the recirculating medium. The HFB maintains cells at densities of >10\(^9\) red blood cells (RBCs)/mL. A microprocessor-controlled pump can circulate medium inside fibers at different rates. We used a rate of 75 mL/min. Cartridge, pump, and bottle with medium were kept inside a gas-tight box filled with 5\% CO\(_2\), 5\% O\(_2\), and 90\% N\(_2\) maintained at 37\textdegree C by a standard incubator.

The medium was changed once or twice a day, depending on the day of the experiment and the culture hematocrit. Samples of cells and medium were collected daily after gentle cell resuspension. Cells were also resuspended at the beginning and end of the day by cartridge rotation. Unless noted, experiments lasted 4 or 5 days without addition of new RBCs. Cells were harvested by flushing the cartridge with medium and were counted by cell counter (Z series; Beckman Coulter). Parasitemia was assessed as described above, and glucose consumption was assessed by biochemistry analyzer (YSI 2700 Select; YSI Life Sciences). Synchronization of \textit{P. falciparum} culture was...
done as described in [8]. Cartridges were used continuously for months.

**Results.** To test whether HFB is suitable for study of *P. falciparum* replication at RBC densities typical for human blood, we performed 6 experiments with *P. falciparum* culture at 45%–50% hematocrit. Parasite replication was robust and reproducible inside the HFB (20-kDa MWCO), whereas replication was minimal in control flask cultures at the same hematocrit (figure 1A). In a subsequent set of 7 experiments, we used RBC densities greater than normal, as observed in some diseases such as polycythemia (60%–80% hematocrit) [9]. Furthermore, to mimic RBC sequestration in patients with cerebral malaria, we tested 100% hematocrit or packed blood (*n* = 2).

We found that *P. falciparum* replicated equally well at all tested cell densities in HFB (table 1). No significant correlation between hematocrit at a range of 20%–100% and parasitemia on day 4 or 5 was found (*P* = .16, *r*² = 0.13, *n* = 16; Pearson correlation analysis; Prism software). Glucose deficiency was prevented by providing 0.24–0.29 mL of medium per day per 8 × 10⁷ cells in culture (cell number in 1 mL of suspension with 1% hematocrit). This level of medium supply kept the glucose concentration in the medium ≥75% of control throughout the experiment (data not shown). Substitution of 0.5% AlbuMax I in the culture medium with 10% human plasma (group A–positive blood donors) did not increase the efficiency of parasite growth (figure 1B). The growth ratio (ratio of final parasitemia:starting parasitemia for 4 or 5 days in culture) was comparable with those reported elsewhere [10–15] for other methods (growth ratio range, 14–35; table 1). This parameter inversely correlated with the starting parasitemia, a phenomenon observed with other methods of *P. falciparum* culture [15].

To test for alterations in plasmodial physiology during culture in an HFB, we took samples from a 4-day-old HFB culture at 60% hematocrit and from a control flask culture at 6% parasitemia.

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**Figure 1.** Kinetics of *Plasmodium falciparum* replication in a hollow-fiber capillary bioreactor (HFB). A, Comparison of *P. falciparum* replication in HFB and in a control flask culture at 45% hematocrit. B, *P. falciparum* replication in HFB at 60% hematocrit. Culture was synchronized as described elsewhere [8]. Culture medium was supplemented with 0.5% AlbuMax I or with 10% human plasma. Culture synchronization was maintained for ∼2 cycles, similar to the Trager-Jensen method [3]. Thus, a high hematocrit is unlikely to be a synchronization factor for the *P. falciparum* life cycle observed in vivo. C, Comparison of replication capacity of *P. falciparum* cultures from HFB and a control flask. After 4 days in culture, cells maintained in HFB at 60% hematocrit and cells from a control flask culture at 6% hematocrit were split with new red blood cells (RBCs) to 0.5% parasitemia at 2% hematocrit and cultured in a 24-well plate for 4 days. D, Effect on *P. falciparum* replication in HFB with or without AlbuMax I at 70% hematocrit (representative experiment). Cells were suspended in 0.5% AlbuMax I in the noncirculating cellular compartment. E, Multiple harvests of parasitized RBC from HFB at 80% hematocrit. Culture was split on day 4 with normal RBCs at 100% hematocrit; 2 more harvests were made on days 6 and 7 of culture. F, Continuous culture of *P. falciparum* in HFB at 50% hematocrit over the course of 16 days. Inset, Cell suspension from HFB after passage 4 was split with normal RBCs to obtain 0.5% parasitemia at 2% hematocrit. This culture was placed in a 24-well plate in parallel with control culture with the same starting parameters.
hematocrit. Both samples had comparable kinetics of replication (figure 1C). Thus, parasite quality did not appear to suffer in this technique.

Independence of parasite growth from hematocrit in HFB could be attributed to a putative negative growth regulatory factor secreted by parasitized erythrocytes that was accumulated inside the bioreactor with the growth of the parasites. To test this hypothesis, 2 other types of cartridges (5-kDa MWCO and 0.1-μm pores) were also tested. *P. falciparum* replication was less efficient in these devices than in 20-kDa MWCO cartridges. On average, 72% of control parasitemia (20-kDa MWCO cartridge, *n* = 3) was observed with 5-kDa MWCO cartridges (*n* = 2) at 80% hematocrit. The 0.1-μm pore cartridge gave comparable levels of plasmidum replication, but the much fewer fibers in this type of cartridge prevented direct comparisons.

An attempt to exclude AlbuMax I from the circulating medium with its preservation within the cartridge (*n* = 2) did not abrogate, but did reduce parasite growth after the first cycle of parasite propagation (figure 1D), presumably because consumption of low-molecular-mass molecules from the complete medium by parasites. Thus, this method may facilitate the identification of these molecules.

In the search for mechanical factors affecting parasite replication at high hematocrit, we compared parasite growth in static and episodically rotated bioreactors. We found a small positive effect of episodic cartridge rotation on parasite growth in experiments with high hematocrit. In 6 experiments with different hematocrit performed with 3 rotations a day, parasitemia reached a 8.5% ± 0.28% (mean ± SE) at days 4–5. However, in 7 experiments done with 1 or 2 rotations a day, the level of parasitemia was significantly lower: 5.94% ± 0.43% (mean ± SE; *P* < .001, by Student’s *t* test; Sigma Plot). We verified these data by performing 1 experiment with 50% hematocrit without cartridge rotation. Parasitemia reached 6% for 5 days with a growth ratio of 12, near that seen with the standard method [3]. Thus, a small promotion of parasite growth is observed in experiments with limited RBC mixing, but replication is substantial without RBC mixing.

We calculated the yield of parasitized cells in 20-kDa HFB cultures (table 1) on the basis of the parasitemia and the number of RBCs in suspension at different hematocrit levels. The yield in 4 or 5 days was comparable with the best reported in the literature for an elaborate and expensive flow vessel when the parasitemia was 20% with 2% hematocrit [15]. Between 37- and 81-fold more parasitized erythrocytes can be obtained routinely from an HFB than from a control T25 flask culture. Moreover, splitting the culture with fresh, normal RBCs further increases the yield of parasitized cells. Figure 1E represents 1 of 2 experiments we performed for this purpose. The yield of parasitized cells in this experiment was 2.0 × 10⁹ cells/week.

To mimic static conditions for RBCs in places of sequestration in vivo and to evaluate the biologic parameters of this culture at the end of passage 4, we performed 16-day experiments in an HFB with a minimal cell mixing. Figure 1F shows the growth of parasite in the HFB at 50% hematocrit. Reproducible growth kinetics was observed from passage to passage. We did not find any sign of culture deterioration: HFB culture under the Trager-Jensen condition of growth, side by side with control culture, demonstrated the similar kinetics of replication (figure 1F, inset). We also did not find significant differences between fractions of multiple parasitized RBCs in these 2 cultures. The mean ± SD percentage of double- and triple-infected cells in HFB culture (*n* = 4) was 21.3% ± 2.5% and 3.2% ± 1.0%, respectively. In control cultures (*n* = 4) these parameters were 17.5% ± 4% and 2.8% ± 2%. Student’s *t* test showed no significant difference between the proportion of multiple parasitized cells in control and HFB cultures (*P* = .17 for double infection and *P* = .69 for triple infection). Thus, sequestered parasitized RBC in vivo may produce healthy progeny of parasites indefinitely.

### Discussion.

The density limitation in the classic Trager-Jensen method of malaria parasite culture is likely due to the deficiency of nutrition; gas exchange requires a thin layer of culture medium (2–4 mm in depth). To avoid this limitation, we used an HFB, as has been used for the propagation of different cell lines at high cell densities [4–7]. The HFB uses multiple hollow fibers to create a semipermeable barrier between the areas of cell growth and areas of medium perfusion.
The hollow fibers provide a large surface area for metabolites and gas and nutrition exchange, which are much needed for efficient parasite growth. Our results show that *P. falciparum* replicates efficiently—even at the highest erythrocyte densities (packed RBC) in an HFB.

One drawback of the HFB method, compared with the continuous flow method, is a diminished limiting parasitemia (8%–12% vs. ~20%, respectively). As discussed, we suspect this difference is due to the accumulation of factors released during culture that have a negative impact on parasite replication. Our preliminary data indicate that medium from inside cartridges with infected cultures has several extra protein bands (range, 74–92 kDa) in comparison with medium from cartridges with normal RBCs. One can use HFB with different fiber pore sizes (MWCO: 5 vs. 20 kDa vs. 0.1-μm pores) to analyze the effect of substances with different molecular masses on parasite replication and to collect small potential immunogens from the circulating media. HFB also allows the accumulation of larger parasite exoantigens inside cartridges for analysis. Neither the standard flask nor the flow chamber cultures facilitate these goals. In addition, the medium change technology of the HFB minimizes the risk of culture contamination.

The most obvious advantage of this method is the simplicity in obtaining high numbers of parasitized RBCs. The HFB method requires approximately one-half as much medium and gas, 3-fold less laboratory space, and 5-fold less labor time than the standard flask culture method. HFB are commercially available. Reuse of cartridges and long-term use of the pump offset the cost of flasks. A moderate parasitemia results in cultures that look clean and healthy. Thus, the HFB method could be used for analysis and preparative isolation of plasmodial factors for vaccine development. Coculture of parasitized RBCs with endothelial cells in a specially designed bioreactor may probe the interplay between these 2 types of cells in the pathogenesis of severe malaria.

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**References**