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# Small-scale culture of *Plasmodium falciparum* using μ-Slide Angiogenesis followed by automatic infection rate counting to assess drug effects☆

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ABSTRACT

It is very important to reduce the costs involved in malarial drug development by small-scale culture of *Plasmodium falciparum*, and automation of the assay system for drug efficacy against the parasites for high-throughput screening. In this study, we report that *P. falciparum*-infected erythrocytes can be stably cultured on μ-Slide Angiogenesis, which is used to investigate angiogenesis in tube formation assays, followed by automatic counting of the infection rate (parasitaemia). After 10 μL of parasite-infected erythrocytes were added to the inner well of μ-Slide Angiogenesis to prevent a multilayer of erythrocytes, 30 μL of silicon oil was overlaid on the culture medium to avoid evaporation of the medium, leading to stable small-scale parasite cultivation. The parasites were stained with a cell-permeant fluorescent nucleic acid stain (SYTO21) followed by cultivation. After taking bright field and fluorescent images using an inverted microscope, the infection rate could be calculated automatically by counting the number of erythrocytes and parasites using MetaMorph Offline software. The effect of anti-malarial drugs on parasite growth could be investigated on μ-Slide Angiogenesis, in which the parasite culture was added to the inner wells containing the drugs followed by their cultivation. Taken together, this method may be useful for image-based screening for anti-malarial drug candidates with automatic counting of parasite infection rates.

## 1. Introduction

Malaria is a mosquito-borne infectious disease caused by the parasitic protist, *Plasmodium* spp., which parasitizes red blood cells (RBCs), resulting in the occurrence of fever and/or anaemia. Malaria caused by *P. falciparum* is the most serious with high mortality. Artemisinin-based combination therapies (ACTs) are recommended by WHO as the first-line treatment for uncomplicated *P. falciparum* malaria [1], but *P. falciparum* at the Cambodia-Thailand border has acquired resistance to ACTs [2]. Considering a risk that multidrug resistance can emerge, assessment of the degree of resistance against existing malarial drugs and development of new anti-malaria drug candidates are very important to control and eliminate this fatal disease.

Conventional standard methods for drug susceptibility testing involve microscopic examination of Giemsa-stained RBC smears or radiometric assays measuring the incorporation of [$^3$H]-hypoxanthine by the parasite [4]. The growth of parasites is presented as infection rate or parasitaemia in the former method and as the amount of nutrient incorporation by parasites in the latter. Since Giemsa-stained images have to be analyzed manually, the former method is laborious. Instead of [$^3$H]-hypoxanthine, non-radioactive methods using the intercalation of DNA with dyes such as SYBR green or PicoGreen, have also been developed [5,6]. Enzyme-linked immunosorbent assay (ELISA) measuring histidine-rich protein II, or ELISA and colorimetric assays measuring the parasite lactate dehydrogenase are also performed to evaluate parasite growth [7–9]. All these assays except for microscopic examination, measure the total signal intensity output and are thus suited for small-scale and high-throughput screening [10–12].

Further, to improve the sensitivity of the assay, image-based analysis of *P. falciparum* using 4′,6-diamidino-2-phenylindole (DAPI) for

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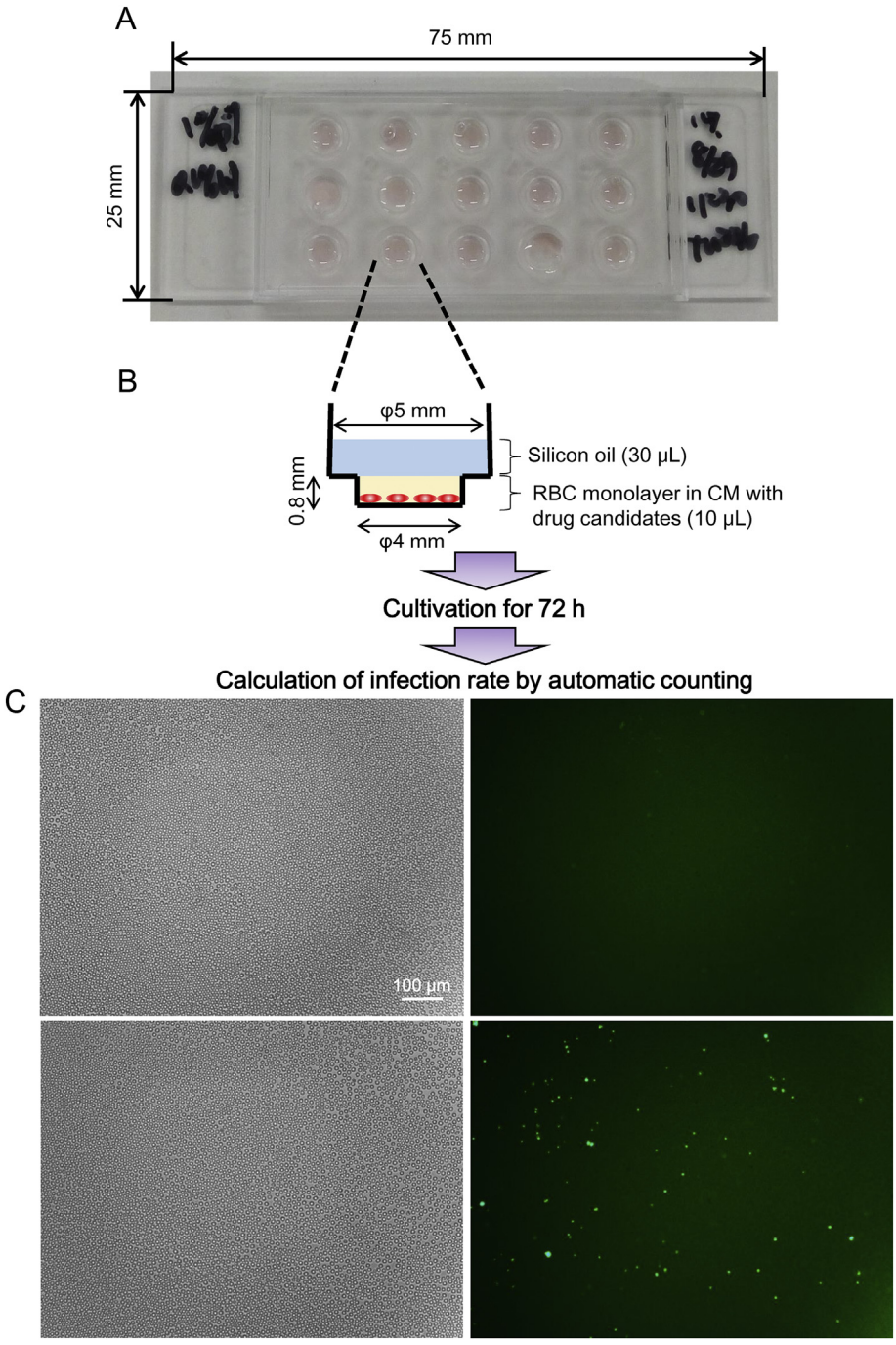


**Fig. 1.** Protocol for *P. falciparum* culture on μ-Slide Angiogenesis. (A) Picture of a μ-Slide Angiogenesis. Parasite cultures overlaid with silicon oil are contained in each well. (B) Schematic presentation of a sectional view of the well. Each well consists of an inner well and an upper well. Medium containing iRBCs (10 μL) was added to the inner well, and silicon oil (30 μL) was added to the upper well. The parasites were cultured for 72 h in medium with or without drugs, followed by automatic calculation of the infection rate. (C) Representative bright field images of RBCs on μ-Slide Angiogenesis after 72 h cultivation (left panels), and fluorescence images of the same field (right panels). Images of the parasite-infected or uninfected RBCs stained with SYTO21 are shown in the upper and lower panels, respectively.

nucleic acid staining was developed [10,13]. The advantage of image output is that specific isolated areas of fluorescence (parasite detection) can be detected allowing the subtraction of all non-parasite-related background readings. In the image-based analysis with 96-well or 384-well culture plates, DAPI-positive parasites are counted for evaluating parasite growth, but infection rate (or parasitaemia) cannot be calculated because RBCs do not form a monolayer at the bottom of the wells and cannot be counted automatically.

In this study, we report a method for small-scale *P. falciparum* monolayer culture followed by automatic counting of the infection rate, thus allowing assessment of drug effects on parasites. For the parasite culture, we used a product developed for studying angiogenesis. This novel image-based analysis method may enable researchers to obtain more reliable data on drug resistance or the effect of novel drug candidates on parasite growth.

## 2. Materials and methods

### 2.1. Plasmodium culture on 48-well plate, 384-well plate, or μ-Slide Angiogenesis

Culture of *P. falciparum* strain 3D7 was performed as described previously [14]. Asynchronous cultures (> 70% ring form) of infected RBCs (iRBCs) were added to 48-well or 384-well plates (Corning Inc., Corning, NY), or μ-Slide Angiogenesis (ibiTreated, ibidi GmbH, Martinsried, Germany) with or without drugs, followed by overlay with silicon oil (Merck & Co. Inc., Kenilworth, NJ). Cultures were incubated at 37 °C under an atmosphere of 5% $O_2$, 5% $CO_2$, and 90% $N_2$.

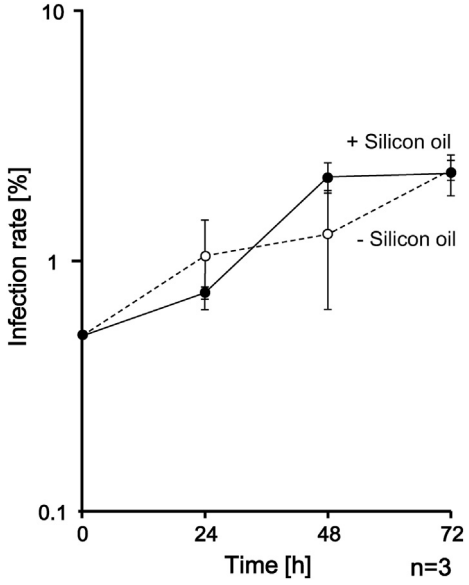


**Fig. 2.** Effect of silicon oil overlay on the parasite growth. iRBCs (0.5% infection rate, 0.1% haematocrit) overlaid with silicon oil were cultured for 72 h in a 48-well plate (closed circles). As a control, a culture without silicon oil was also examined (open circles). Infection rate was investigated every 24 h by microscopic examination of Giemsa-stained blood films.

### *2.2. Automatic Plasmodium infection rate counting*

Counting infection rate after cultivation of parasites on μ-Slide Angiogenesis was performed as described previously [15]. Briefly, the culture medium and silicon oil were removed from the well using a pipette; the RBCs remained and formed a monolayer on the hydrophilic-treated bottom of the well. Next, 50 μL of RPMI 1640 containing the fluorescent nucleic acid staining dye, SYTO21 (final concentration 5 μM, Thermo Fisher Scientific, Waltham, MA) was added and incubated for 10 min. Bright field and fluorescence images of parasite-infected RBCs (iRBCs) stained with SYTO21 were acquired using an inverted fluorescence microscope (DM1L, Leica Microsystems, Wetzlar, Germany) with a digital camera (MC120 HD, Leica Microsystems). MetaMorph Offline software (ver. 7.8, Molecular Devices, Sunnyvale, CA) was used for automatic counting of the number of RBCs and infected parasites, followed by calculation of the infection rate from the acquired microscopic images [15].

### *2.3. Plasmodium culture on μ-Slide Angiogenesis with anti-malarial drugs*

Chloroquine Diphosphate (CQ, Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) or dihydroartemisinin (DHA, Tokyo Chemical Industry Co. Ltd.) was properly diluted with dimethyl sulfoxide (DMSO). Next, 2 μL of the diluted anti-malarial drug was applied to the bottom of an inner well of the μ-Slide Angiogenesis. By incubating at 25 °C for > 10 min, the DMSO was evaporated and only CQ or DHA remained in the well. Cultivation of the parasites and automatic infection rate counting was then performed as described above.

## 3. Results and discussion

### *3.1. Methods for small-scale culture of P. falciparum on μ-Slide Angiogenesis*

It is difficult to obtain equipment or chemicals for drug resistance assays of parasites in the field setting of endemic areas. Further, screening for drug candidates is required to develop novel drugs. Therefore, small-scale cultures to investigate drug effects on parasite growth need to be performed for both drug resistance assays and drug development. For image-based assays, 96-well or 384-well plates are used for parasite culture with drugs or drug candidates, and 100 μL or 25 μL of culture medium is required for each well, respectively [10,11]. We attempted to culture parasites on μ-Slide Angiogenesis, which was developed for angiogenesis research, and consists of 15 wells (Fig. 1A). Each well consists of an inner well and an upper well (Fig. 1B); 10 μL of the gel matrix required for angiogenesis is added to the inner well followed by overlaying with 50 μL of cell suspension added to the upper well. Stable small-scale culture and microscopic examination for angiogenesis can be performed using these slides.

We cultured *P. falciparum* in the inner wells (Fig. 1B). Ten microliters of culture (0.1% haematocrit) was added to the inner wells, resulting in the formation of an RBC monolayer on the bottom of the wells. To avoid evaporation of the culture medium, 30 μL of silicon oil was added to the upper wells to overlay the culture medium. Recently, we reported a method for preparing RBC monolayers on hydrophilic-treated plastic plates followed by automatic counting of the parasite infection rate [15]. The μ-Slide Angiogenesis used in this study have already been hydrophilic-treated.

After cultivation, the medium and silicon oil were removed with a pipette, and medium containing SYTO21 was added to the RBCs and incubated for 10 min. The parasites infecting the RBC monolayer at the bottom of the well were stained with the fluorescent dye. Bright field images and fluorescence images were taken using an inverted fluorescent microscope with a digital camera (Fig. 1C). These images were analyzed to calculate the infection rate using MetaMorph Offline software [15].

## 4. Overlay of silicon oil on the culture does not disturb *Plasmodium* growth

For stable small-scale culture, it may be important to avoid alteration of medium components by evaporation. Silicon oil has low cytotoxicity and high gas permeability. Therefore, the effect of silicon oil overlay on parasite growth was investigated (Fig. 2). iRBCs (250 μL, 0.5% infection rate, 0.1% haematocrit) were added to a well of a 48-well plate, overlaid with 250 μL of silicon oil, and cultured for 72 h. The infection rate was examined every 24 h by microscopic examination of Giemsa-stained blood films. As a control experiment, cultures without silicon oil were also analyzed. During the culture period, parasites were able to grow normally with the silicon oil overlay.

### *4.1. Plasmodium culture on μ-Slide Angiogenesis*

*P. falciparum* culture was performed on μ-Slide Angiogenesis followed by automatic infection rate counting (Fig. 3A). As control experiments, iRBCs (0.5% infection rate, 0.1% haematocrit) were cultured on 48-well or 384-well plates. For 48-well plates, 250 μL of culture was overlaid 250 μL of silicon oil. For 384-well plates, 10 μL of culture was overlaid with 30 μL of silicon oil. The plates were incubated for 72 h, and the infection rate was investigated every 24 h by microscopic examination of Giemsa-stained thin blood smears. Ten μL of iRBC culture (0.5% infection rate, 0.1% haematocrit) was added to the inner well of a μ-Slide Angiogenesis followed by 30 μL of silicon oil added to the upper well for overlaying the culture medium.

On the other hand, for the culture in the μ-Slide Angiogenesis, the culture was incubated for 72 h, and infection rate was automatically investigated every 24 h by SYTO21 staining followed by analysis with MetaMorph Offline software. The growth of parasites on the μ-Slide Angiogenesis was similar with that on 48-well or 384-well plates. Therefore, these results indicate that parasites could grow normally on μ-Slide Angiogenesis and that automatic counting of the infection rate could be performed properly.

Next, we investigated effect of infection rate at the beginning of cultivation on the parasite growth on μ-Slide Angiogenesis (Fig. 3B).

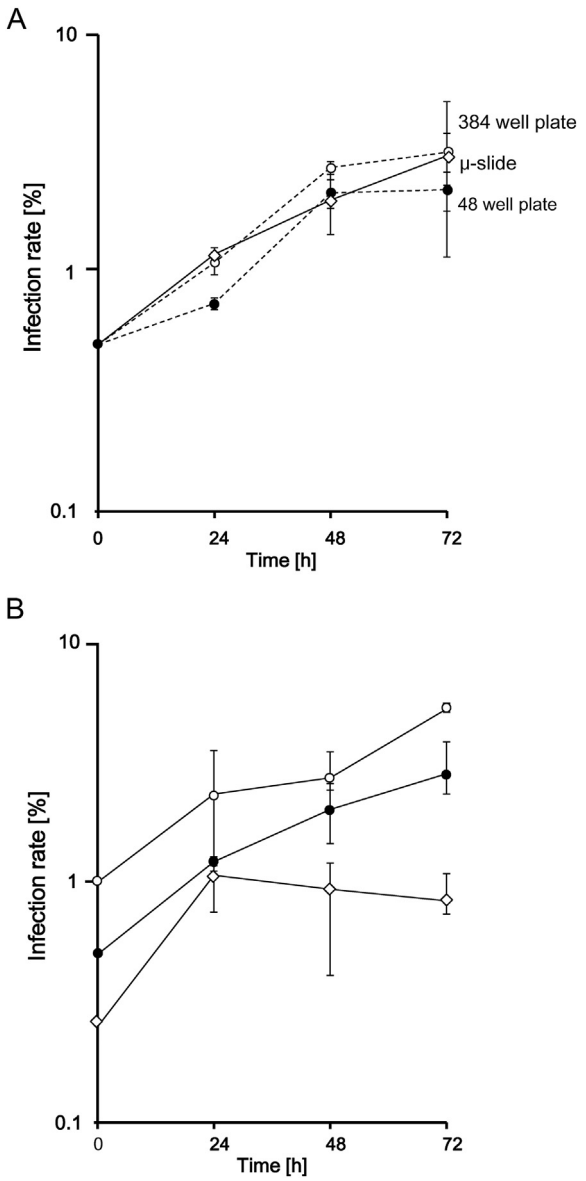


**Fig. 3.** *Plasmodium* culture on μ-Slide Angiogenesis. (A) *P. falciparum* was cultured on 48-well plates (closed circles), 384-well plates (open circles), or μ-Slide Angiogenesis (diamonds). iRBCs (0.5% infection rate, 0.1% haematocrit) were cultured for 72 h in each well, and the parasite growth were compared. (B) Growth curves of the parasite culture for 72 h on μ-Slide Angiogenesis are shown. The infection rate at the beginning of cultivation was 1.0% (open circles), 0.5% (closed circles), and 025% (triangles).

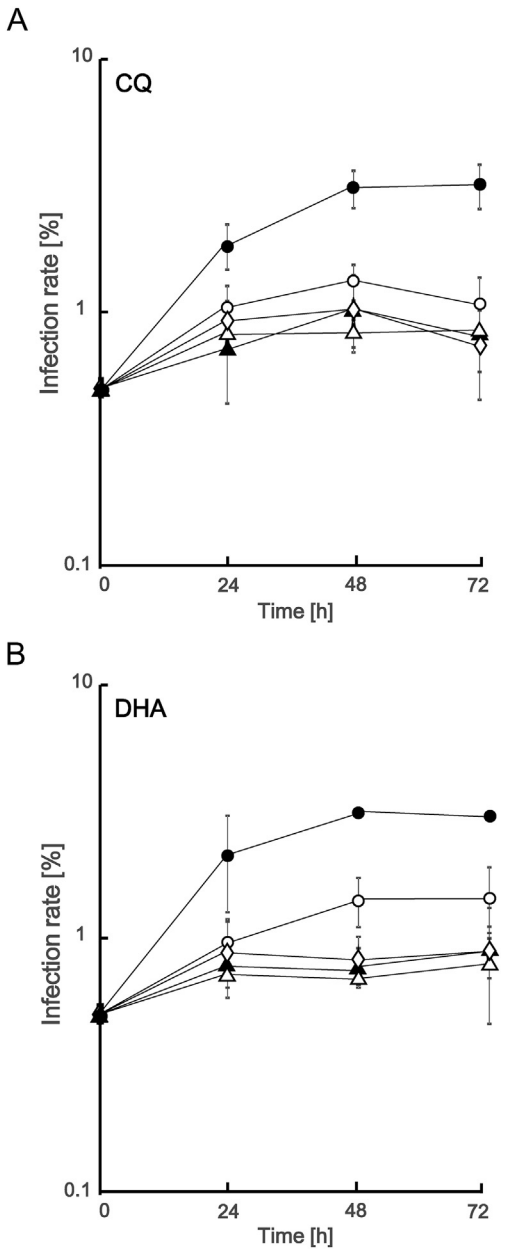


**Fig. 4.** Growth inhibition of *Plasmodium* culture on μ-Slide Angiogenesis by anti-malarial drugs. *P. falciparum* was cultured on μ-Slide Angiogenesis with 0 (closed circles), 1.6 (open circles), 25 (open triangles), and 100 nM (open diamonds) of CQ (A), or with 0 (closed circles), 0.2 (open circles), 4 (closed triangles), 80 (open triangles), and 160 nM (open diamonds) of DHA (B). The iRBCs (0.5% infection rate, 0.1% haematocrit) were cultured for 72 h, and infection rate was investigated every 24 h.

iRBCs with several infection rates (0.25%, 0.5%, or 1.0%) were cultured for 72 h. The infection rate was investigated every 24 h. As the growth of parasites with an initial infection rate of 0.25% was very slow after 24 h cultivation, the initial infection rate should be > 0.5%.

### *4.2. Evaluation of growth inhibition in Plasmodium cultured on μ-Slide Angiogenesis by anti-malarial drugs*

We examined whether growth inhibition of parasites by anti-malarial drugs could be investigated using parasite cultured on μ-Slide Angiogenesis followed by automatic infection rate counting (Fig. 4). *P. falciparum* (0.5% infection rate, 0.1% haematocrit) was cultured for 72 h with various concentrations of CQ (Fig. 4A) or DHA (Fig. 4B), and the infection rate was automatically counted every 24 h. The parasites without anti-malarial drugs grew normally (closed circles in Fig. 4A and B). The growth of parasites was strongly inhibited by 1.6 nM CQ or 0.2 nM DHA. The parasites could not grow in the presence of > 6.3 nM CQ or 4 nM DHA.

Previously, it has been reported that the *in vitro* 90% inhibitory concentration ($IC_{90}$) for the 3D7 strain was 21.5 ± 6.6 nM for CQ [16], and 2.11 ± 0.12 nM for DHA (). In this study, the parasites could hardly grow in the presence of 25 nM CQ or 4 nM DHA, indicating that the anti-malarial agents worked properly against the parasites on μ-Slide Angiogenesis, and that the effect on the infection rate could be monitored. Taken together, the drug effect on the parasite infection rate can be examined easily by this method when compared with the microscopic analysis of Giemsa-stained blood smears, which may contribute to establish more reliable anti-malarial drug screening methods.

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