Published in final edited form as:

Mol Biochem Parasitol. 2017 December ; 218: 16–22. doi:10.1016/j.molbiopara.2017.10.001.

Comparison of two methods for transformation of *Plasmodium knowlesi*: direct schizont electroporation and spontaneous plasmid uptake from plasmid-loaded red blood cells

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Abstract

Human infections from *Plasmodium knowlesi* species present challenges to malaria control in Southeast Asia. *P. knowlesi* also offers a model for other human malaria species including *Plasmodium vivax*. *P. knowlesi* parasites can be cultivated in the laboratory, and their transformation is standardly performed by direct electroporation of schizont-infected red blood cells (RBCs) with plasmid DNA. Here we show that the efficiency of direct electroporation is exquisitely dependent on developmental age of the schizonts. Additionally, we show that transformation of *P. knowlesi* can be achieved without direct electroporation by using the parasite's ability to invade and take up DNA from plasmid-loaded RBCs. Transformation with plasmid-loaded RBCs does not require labor-intensive preparations of schizont-infected RBCs as for direct electroporation, and parasite damage from high voltage discharge is avoided. Further studies of the mechanism of spontaneous DNA uptake may suggest strategies for improved transformation and provide insights into the transport pathways of apicomplexans.

Keywords

malaria; transfection; cell electroporation; luciferase; genetic modification

1. Background

Plasmodium knowlesi, a natural parasite of macaque monkeys, is now a major cause of malaria in Malaysia, where 10% of the clinical cases it causes are considered severe and 1–

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Competing Interests

The authors of this manuscript declare no competing interests.

Authors' Contributions

RMB, TJG and TEW designed studies and wrote the manuscript. RMB, TJG, WAK, and JMS performed experiments and collected data. All authors contributed revisions and approved the manuscript.

2% result in death [1–3]. Another 8 countries in Southeast Asia report *P. knowlesi* infections [1], a fact that expands the agenda of malaria control efforts largely focused on the elimination of *P. falciparum* and *P. vivax* [4].

In addition to its importance as an emerging pathogen, *P. knowlesi* provides an important model for drug and vaccine research, and it provides a system for studies of other malaria parasite species that are less tractable for experimental investigation [5, 6]. An example is the *P. vivax* species, a leading cause of human malaria in the same evolutionary clade as *P. knowlesi* [7, 8]. While methods for effective *in vitro* cultivation of *P. vivax* remain unavailable, *P. knowlesi* cultures in human as well as rhesus macaque red blood cells (RBCs) are useful for a range of experimental studies [9–11]. The full *P. knowlesi* life cycle can be supported through rhesus macaques and *Anopheles* mosquitoes in the laboratory, and genetic transformation of *P. knowlesi* parasites can be achieved *in vivo* and *in vitro* [9, 12–14].

P. knowlesi transformation methods have relied on the direct electroporation of infected RBCs [9, 15]. Likewise, transformation of *P. falciparum*-infected RBCs was performed only by direct electroporation until the development of an alternative method that took advantage of spontaneous DNA uptake by parasites in plasmid-loaded RBCs [16]. Comparative studies showed that the spontaneous uptake method is both convenient and efficient [17], and it has served for manipulations and knock-outs of genes that affect important phenotypes, including antigenic variation, drug resistance and parasite invasion of RBCs [18–21]. We now report successful transformation of *P. knowlesi* by DNA uptake from plasmid-loaded rhesus RBCs, and we compare the results to the outcomes of transformation by electroporation of schizont-infected RBCs.

2. Methods

2.1 Plasmid constructs

Plasmid pHDEF1i-luc (Fig. 1A) contains the firefly luciferase cassette (*fLuc*) under control of a 1.4 kb 5′ untranslated region (UTR) from the *Plasmodium berghei* elongation factor 1- α gene (*pbef1- α*) and a 0.6 kb 3′ UTR from the *P. falciparum* histidine rich protein 2 gene (*pfhrp-2*). In previous work, the *pbef1- α* 5′ UTR was used to promote expression of *fLuc* in *P. knowlesi* [13]; it also served as a bidirectional promoter to drive the simultaneous expression of the human dihydrofolate reductase gene (*hdhfr*) and a *fLuc* cassette in opposite directions [19].

Plasmid pD-PfCam-luc (Fig. 1A) was generated by replacing the *pfCRT* gene in plasmid pDC/CRT-FLAG [22] with the *fLuc* cassette, between the XhoI restriction enzyme sites. It thus carries the *fLuc* cassette under control of a 0.6 kb 5′ UTR sequence from the *P. falciparum* calmodulin gene (*pfcam*) and a 0.8 kb 3′ UTR sequence from *P. falciparum* heat shock protein 86 (*pfhsp86*). pD-PfCam-luc also contains the *hdhfr* cassette under control of a 0.6 kb 5′ UTR from the *P. chabaudi* *dhfr-ts* gene (*pcdts* 5′), and a 0.8 kb 3′ UTR from *pfhrp2*.

2.2 Rhesus red blood cells and *P. knowlesi* cultures

Rhesus blood was obtained according to the NIH Guidelines for Animal Care and Use, under an Animal Study Proposal approved by the NIAID Animal Care and Use Committee (ACUC). Samples were collected into vacutainer tubes coated with sodium heparin (BD, Franklin Lakes, NJ, USA) and centrifuged at $800 \times g$ for 3 min; plasma was removed and the RBCs were washed once with incomplete RPMI (iRPMI, consisting of 0.26% sodium bicarbonate in RPMI-1640 (with 25 Mm Hepes and 50 $\mu\text{g}/\text{ml}$ hypoxanthine, KD Medical, Columbia, MD, USA)). After the wash, the RBCs were suspended to a final 50% hematocrit (hct) in complete RPMI (cRPMI, consisting of iRPMI supplemented with 10 mg/L gentamicin, and 1% Albumax II (Life Technologies, Carlsbad, CA, USA)). This RBC suspension was stored as stock at 4°C and used within 2 weeks of processing.

P. knowlesi H strain cultures were initiated from thawed cryopreserved stocks gifted by Dr. Sanjay Desai (LMVR, NIAID). The cultures were maintained in cRPMI with rhesus RBCs at 5% hct, under a 90% N₂/5% CO₂/5% O₂ gas mixture at 37 °C with daily media changes. Parasite development and propagation through the *P. knowlesi* 24-hour asexual blood stage cycle was monitored by microscopy of thin blood films fixed in methanol and stained for 15 min with 20% Giemsa (Sigma-Aldrich, St. Louis, MO, USA). Parasitemias were estimated by counting the number of parasitized RBCs (pRBCs) per 1,000 RBCs. Cultures were maintained with parasitemias between 0.5 and 10.0%. Parasitemias increased ~3–5-fold/day in Albumax supplemented RPMI (cRPMI), similar to previous reports of *P. knowlesi* growth in serum supplemented RPMI [9, 10].

2.3 Percoll gradient for schizont enrichment

Synchronized *P. knowlesi* cultures and schizonts were obtained by a Percoll step-gradient modified from that described for *P. falciparum* [23, 24]. Briefly, cultures containing > 3% segmented schizonts were centrifuged at $800 \times g$ for 3 min, and a 300 μL pellet containing the pRBCs was resuspended in 3 mL of cRPMI. This mixture was gently placed onto a 35%/65% Percoll (GE Healthcare Life Sciences, Pittsburgh, PA, USA) step-gradient and centrifuged ($1500 \times g$, 15 min, no brake). Late-stage schizonts were collected in a 500 μL volume at the gradient step and centrifuged at $800 \times g$ for 3 min. The recovered schizonts (20 μL volume) were washed once with 5 mL of cRPMI.

2.4 Transformation of *P. knowlesi* by electroporation of schizont-infected RBCs

Direct electroporation of *P. knowlesi* schizont-infected RBCs was performed as described by Janse et al. [15] and Moon et al. [9]. For each electroporation experiment, a total of 1×10^7 or 3.5×10^7 schizont-infected RBCs was purified by Percoll gradient from a 20 mL synchronized *P. knowlesi* culture (5% hct). The recovered cells (up to 50 μL) were suspended in 100 μL of P3 primary cell solution (Lonza, Basel, Switzerland) and mixed with 20 μg of plasmid DNA in 10 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). This suspension was transferred to a 4D Nucleofector XL cuvette and subjected to electroporation in the Lonza 4D Nucleofector System (Lonza), using program FP158. Immediately after electroporation, the suspension was placed into a 2 mL tube containing 500 μL of cRPMI and 300 μL of stock 50% hct rhesus RBCs. After 30 min at 37°C in a shaking incubator (250 rpm), the mixture (900 μL total) was transferred to a 25 cm² vented culture flask (Thermo

Fisher, Waltham, MA, USA) with 5 mL of fresh cRPMI under a 90% N₂/5% CO₂/5% O₂ gas mixture. For drug selection experiments, an additional 200 µL of fresh 50% hct rhesus RBCs was added to cultures. Cells were collected 48 h later for luciferase assays or maintained in culture for selection under 1 nM WR99210 pressure (Fig. 1B).

2.5 Transformation of *P. knowlesi* by DNA uptake from plasmid-loaded rhesus RBCs

For plasmid loading, 300 µL of pelleted rhesus RBCs were washed twice by centrifugation and resuspension in 5 mL cytomix (120 mM KCl, 0.15 mM CaCl₂, 2 mM EGTA, 5 mM MgCl₂, 10 mM K₂HPO₄/KH₂PO₄, 25 mM Hepes, pH adjusted to 7.6 with KOH) [25]. The RBCs were recovered, placed in a 1.5 mL microcentrifuge tube with 100 µL cytomix containing 40 µg of plasmid DNA, and transferred to a 0.2 cm electroporation cuvette (Bio-Rad, Hercules, CA, USA). Electroporations of the uninfected RBCs were performed in a Bio-Rad Gene Pulser II at 310V and 975 µF, with resulting time constants of 20–35 ms. The plasmid-loaded RBCs were washed twice with 5 mL of iRPMI and used immediately.

For transformation of parasites by spontaneous DNA uptake, the plasmid-loaded RBCs were added to 10 mL of cRPMI in a 25 cm² flask, for an hct of 2.5%. A concentrated suspension (50 µL) of 3% asynchronous pRBCs was added to introduce a parasitemia of 0.5% (1.5 × 10⁷ pRBCs total) and an hct of 3%. The flask was maintained under culture conditions and, after 24 h, fresh plasmid-loaded RBCs were again added, for a final culture hct of 5% with an estimated 90% of RBCs from plasmid-loaded stock. After another 48 h in culture, cells were collected for luciferase assays (Fig. 1) and/or continued in culture under 1 nM WR99210 pressure.

2.6 Luciferase Assays

To remove hemoglobin and avoid a possible quenching effect of luciferase assay signal, pRBCs were treated with saponin: up to 500 µL of pRBCs were collected from cultures by centrifugation (800 × g, 3 min), overlying supernatant was removed, and the cells were twice suspended and re-pelleted in 0.15% saponin in 1× PBS (10 mM PO₄³⁻, 137 mM NaCl, 2.7 mM KCl, pH 7.4). After washing twice with 1× PBS, the parasites were pelleted, resuspended in 50 µL 1× Cell Culture Lysis Reagent (Luciferase Assay System (Promega, Madison, WI)) and incubated for 10 min at room temperature. The resulting lysate (volume 50 – 60 µL) was cleared by centrifugation (1 min at 10600 × g) and 20 µL of the supernatant (upper liquid layer) was mixed with 100 µL of Promega Luciferase Assay Reagent for luminescence assessment with a GloMax 20/20 Luminometer (10 s integration time). Measurements were collected in technical duplicates and averaged for analysis.

2.7 WR99210 selection of *P. knowlesi* transformants

Parasites transformed by the pD-PfCam-luc plasmid were selected by addition of 1 nM WR99210 to the daily culture media changes (selection attempts failed at the higher levels of 2.5 nM used in another study [9]). Samples from the culture (80 µL packed RBCs volume) were collected for luciferase assays at the time selection was initiated and every 4 days afterwards. To maintain a steady hct, fresh rhesus RBCs were added to replace the cells removed by sampling.

3. Results

3.1 Parasite survival and luciferase activity after transformation by two different methods

To assess parasite survival and growth following transformation by the two methods, we compared total parasite numbers in the cultures before and after transformation with plasmid pHDEF1i-luc. In direct electroporation experiments (SET#1, SET#2, SET#3) a 3-fold decrease was observed, from 3.5×10^7 schizont-infected RBCs electroporated to an average of 1.15×10^7 morphologically intact parasites 48 h later, indicating high mortality after electroporation (Fig. 2A, Table S1). This evidence for extensive parasite damage was accompanied by a high prevalence of parasite debris and pyknotic forms. In contrast, in transformation experiments with plasmid-loaded RBCs (PUT#1, PUT#2, PUT#3), the parasite number increased 22-fold over 72 h, from 1.5×10^7 in the initial inoculum to 33×10^7 parasites. Pyknotic forms and parasite debris were absent from the transformations with plasmid-loaded RBCs.

In three independent experiments, we observed that the average luminescence signal from electroporation of synchronized schizonts was overall greater than from mixed parasite stages transformed by plasmid-loaded RBCs (Fig. 2B). Specifically, 48 h signals (average $\pm$ standard deviation – SD) of $34,659 \pm 883$, $16,612 \pm 1,254$, and $22,790 \pm 161$ LUs were obtained in three experiments with PHDEF1i-luc-electroporated schizonts (Table S2). In comparison, signals of $2,315 \pm 317$, $4,414 \pm 16$, and $1,342 \pm 8$ LUs were obtained from parasites grown for 72 h in PHDEF1i-luc-loaded rhesus RBCs (Table S3).

3.2 Electroporation efficiency varies greatly between developing, nearly mature, and mature schizonts

Morphological differences distinguish developing (early-stage), nearly mature (mid-stage) and mature (late-stage) schizonts of *P. knowlesi* as they progress to merozoite release [26] (Fig. 3). Late-stage segmented schizonts of *Plasmodium berghei* rodent parasites have been stated to be best for transfection and genetic modification [15]. We therefore designed experiments to test the effect of schizont maturity on transformation efficiency of *P. knowlesi* parasites.

P. knowlesi parasites were tightly synchronized by two consecutive Percoll density gradients, allowed to mature through their erythrocytic cycle in the presence of fresh RBCs, and subsequently harvested for schizont samples 18 h, 21 h and 24 h after merozoite invasion (Table 1). After direct electroporation of these developing, nearly mature, and mature schizonts with pD-PfCam-luc, the parasites were returned to separate flasks, maintained under culture conditions, and taken for luminescence measurements precisely 48 h (2 cycles) after reinvasion. Results showed dramatic differences in the signals from the 18 h, 21 h and 24 h schizont preparations (Table 1). In experiment SS#1, luminescence signals (average $\pm$ SD) jumped from $3,620 \pm 126$ LUs in the 18 h preparation to $1,465,131 \pm 581,935$ LUs in the 21 h preparation, before decreasing to $526,487 \pm 21,436$ LUs in the 24 h preparation. In experiment SS#2, luminescence jumped from levels of $1,322 \pm 4$ and $161,190 \pm 4,941$ LUs in the 18 h and 21 h preparations to $4,055,306 \pm 221,242$ LUs in the 24 h preparation. The difference of peak signal times in experiments SS#1 and SS#2 likely

resulted from a few hours difference in the maturity of the schizont preparations. Evidence for this difference is found in the stage distribution of the SS#1 preparation at 24 h, which showed that nearly half of the parasites were young ring forms from merozoite release and reinvasion (Table 1). Young rings forms do not transform efficiently by direct electroporation, likely explaining the decrease of luminescence at 24 h relative to the 21 h preparation.

3.3 Parasite growth and transformant selection using WR99210

To study the recovery of resistant parasites obtained with each transformation method followed by drug selection, we modified *P. knowlesi* parasites with plasmid pD-PfCam-luc, which confers both luciferase expression and WR99210 resistance (Fig. 1A). Transformants were subjected to selection with 1nM WR99210, either 48 h after schizont electroporation or 72 h after the first addition of plasmid-loaded RBCs to parasite culture. Almost all parasites were quickly eliminated from culture so that survivors were rare or undetectable in thin smears four days after application of drug pressure (Fig. 4A, Table S5); however, WR99210-resistant parasites subsequently grew out with each method. In three independent experiments with directly-electroporated schizont-infected RBCs (SE#1, SE#2 and SE#3), parasitemias > 0.01% were detected 8 days after transfection of the 24 h stages, 8 – 12 days after transfection of 21 h stages, and 14 – 18 days after transfection of 18 h stages excepting one culture that remained negative (SE#1 18 h) (Table S5). In two independent transformations by DNA uptake from plasmid-loaded RBC (PU#1, PU#2), parasitemias 0.01% were observed on days 15 and 17 (Table S5). After WR99210 selection, all drug-resistant parasites showed a growth rate of 3 – 5-fold/day, similar to that of un-transfected parasites.

Along with parasitemia counts, luciferase signals of the WR99210-selected transformants were assessed over the course of drug selection (Fig. 4B, Table S6). Luminescence levels obtained 48 h after direct electroporation varied according to the age of schizonts electroporated. Transformants from developing schizonts (18 h stages) in three independent experiments (SE#1, SE#2 and SE#3), presented 48 h luciferase signals (average $\pm$ SD) of 257 ± 12 , $1,177 \pm 38$, and $1,214 \pm 6$ LUs. Transformants from 21 h schizonts presented signals of $41,033 \pm 5,552$, $3,715 \pm 134$, and $22,395 \pm 151$ LUs, whereas the transformants from 24 h schizonts yielded the highest signals, $156,958 \pm 7,870$, $229,884 \pm 5,173$, and $117,033 \pm 483$ LUs. Fig. 4B shows plots of the average signals $\pm$ standard error of the mean (SEM) of the experiments. Following the initial period of WR99210 selection, these signals rose in general accord with the expansion of transformant populations in the cultures; only one experiment (SE#1 18 h) showed no development of luciferase activity (Table S6), consistent with the absence of any microscopically observed transformants as noted above.

In two independent experiments (PU#1, PU#2), transformants obtained by the plasmid uptake method presented 72 h luciferase signals (average $\pm$ SD) of $1,269 \pm 18$ and $1,588 \pm 31$ (Table S6). Signals then decreased 1.6 – 2.5-fold by day 7, after which they rose at a rate similar to that of the transformants from electroporated schizonts (Fig. 4B).

4. Discussion

In comparative studies of *P. knowlesi* transformation methods, our results have led to two findings: (1) transformation efficiency by direct electroporation is highest with late-stage mature schizonts; and (2) stably-transformed *P. knowlesi* parasites can be efficiently obtained by taking advantage of their ability to spontaneously take up DNA from host RBC. DNA uptake from plasmid-loaded RBCs was originally described for *P. falciparum* parasites and has been widely used for the genetic manipulation of these parasites [16, 18, 19, 21, 27]. The success of this method now with *P. knowlesi* suggests that spontaneous DNA uptake from host RBCs is a general property that may support transformation of additional *Plasmodium* species and perhaps some other members of the broad alveolate group.

Direct-electroporation of late (23 – 24 h) *P. knowlesi* schizont stages with *luc* plasmids yielded populations with much greater luciferase activity than did direct-electroporation of younger developing (18 h) schizont stages. Use of mature, well-segmented schizonts thus provided the highest transformation efficiencies, as has been observed also for *P. berghei* [15]. Electroporation of early 18 h stages with pD-PfCam-luc produced signals 100–600-fold lower than similar electroporation of the well-segmented 24 stages; indeed, transformants from the 18 h stages in one case showed no detectable luminescence or survival after WR99210 selection. In transformation experiments involving spontaneous plasmid uptake, results from mixed-stage parasites exposed to pD-PfCam-luc-loaded RBCs were comparable to the successful electroporations of 18h stages, as assessed by yield of transformants and their luciferase signals.

With both transformation methods, intact parasites in thin blood smears decreased to microscopically unobservable levels 4 – 8 days after initiation of WR99210 selection pressure. The period for outgrowth of drug-resistant parasites with both protocols was therefore longer than reported by Moon et al. [9], who observed expanding populations of transformants after 4 days of pressure. Heterologous promoters in our constructs may account for some of this difference, as the selection times of 4 days were observed with markers under the control of endogenous 5' UTRs. Weaker activity of heterologous promoters may require higher plasmid copy number to allow selection of drug resistant parasites, similar to the copy number increase observed in *P. falciparum* transformants subjected to higher drug concentrations [28]. Furthermore, the selection period observed in the spontaneous plasmid uptake method (15 – 17 days, 10^7 pRBC) was similar to reports of direct electroporation of asynchronous cultures using heterologous constructs (two weeks, 10^9 pRBC) [12, 13]. We also note that the interval to select transgenic parasites by the spontaneous plasmid uptake method was about one-half to two-thirds of the time commonly observed for *P. falciparum* [17], in agreement with different blood stage cycle periods of these two species (24 h for *P. knowlesi*; 48 h to *P. falciparum*) [26].

Spontaneous uptake of DNA from plasmid-loaded RBCs offers several advantages of convenience over the schizont electroporation method for transformation of *P. knowlesi*. Stored RBCs can be quickly electroporated with plasmid and simply placed into an ongoing *P. knowlesi* culture for immediate initiation of transformation. An inoculum of 1.5×10^7 asynchronous parasites (50 μ L of packed pRBCs at 3% parasitemia) is sufficient, avoiding

the resource requirements to synchronize parasites by density gradients and prepare large numbers of purified schizonts for electroporation. Transformation by plasmid-loaded RBCs also avoids adverse effects of Percoll density gradients [29] and stresses from electroporation that may affect transient signals used for studies of regulatory sequences.

Despite broad use of spontaneous uptake for *Plasmodium* transformation, the mechanism of plasmid uptake by parasites remains obscure. In single-particle trafficking experiments with fluorescently labeled DNA in CHO cells, electroporation was found to promote DNA/membrane interactions at the cell surface and subsequent entry of plasmid aggregates, possibly in vesicles by endocytosis or directly through electropores [30]. The plasmids were actively transported by fast microtubule-related and slow actin filament-related processes along the cytoplasmic network to the nuclear envelope, where they then crossed into the nucleus and were expressed. Analogous trafficking and expression processes seem likely in *Plasmodium*-infected RBCs; however, DNA/membrane interactions and plasmid incorporation happen spontaneously in these cells without any need for electroporation. Plasmid DNA may be taken up with host RBC contents into parasite cytotomes and phagotrophs, which have been found to convey hemoglobin either directly or via intermediate vesicles to a central digestive vacuole [31] [32]. For expression to happen, DNA in these hemoglobin-containing compartments would have to escape for redirection and entry into the nucleus. Apart from cytotomes and phagotrophs, parasite protein peripherally associated with the parasitophorous vacuolar membrane (*e.g.* *P. falciparum* PfCG2 [33]) may be involved in other processes of host cell cytoplasm uptake into the intracellular parasite. Better understanding these various uptake pathways and their roles in expression of DNA from plasmid-loaded RBCs may lead to improved methods for genetic transformation and provide new insights into parasite transport biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Lynn Lambert, Sachy Orr-Gonzales, and Theresa Engels for providing rhesus blood and technical support. This research was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Disease, National Institutes of Health.

Abbreviations

RBC	red blood cell
pRBC	parasitized red blood cell
hct	haematocrit
dhfr	dihydrofolate reductase gene
hdhfr	human dihydrofolate reductase gene

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Highlights

- *P. knowlesi* parasites transform themselves by DNA uptake from host red blood cells
- Transformation by DNA uptake is less laborious than by direct electroporation
- Efficiency of transformation by direct electroporation varies with age of schizonts
- DNA uptake mechanism studies may give insights into apicomplexan transport pathways

Fig. 1.

Plasmid maps and the timelines of two transformation methods. A The pHDEF1i-luc plasmid contains a firefly luciferase *fLuc* cassette driven by *P. berghei ef1-α* 5' UTR (*pbe1-α* 5'). The pD-PfCam-luc plasmid expresses *fLuc* and contains the *hdhfr* gene that confers resistance to WR99210. The *fLuc* cassette is driven by the *P. falciparum cam* 5' UTR (*pfcam* 5') and the *hdhfr* cassette is driven by the *P. chabaudi dts* 5' UTR (*pcpts* 5'). The arrows indicate directions of transcription; *pfhrp2* 3' and *pfhsp86* 3' indicate 3' UTR terminating sequences from the *pfhrp2* and *pfhsp86* genes. B For transformation by the schizont electroporation method, schizont-infected RBCs were isolated by Percoll gradient, electroporated in the presence of plasmid, and placed into culture for luminescence measurement 48 h later. For transformation by spontaneous DNA uptake, plasmid-loaded RBCs were added to asynchronous pRBCs. Freshly-prepared, plasmid-loaded RBCs were added again at 24 h, and the cells were harvested from the culture at 72 h for luminescence measurement.

Fig. 2. Counts and luciferase activities of parasites transformed by schizont electroporation or spontaneous plasmid uptake

A Number of morphologically-intact parasites in cultures of *P. knowlesi* obtained after direct electroporation of 3.5×10^7 schizont-infected RBCs (left, harvested for luminescence measurement at 48 h) or by spontaneous DNA uptake after the inoculation of 1.5×10^7 asynchronous parasites into plasmid-loaded RBCs (right, harvested at 72 h). The graphs show averages $\pm$ standard errors of the mean (SEMs) from three independent experiments. **B** Luciferase activities of *P. knowlesi* parasites transformed with pHDEF1i-luc by either direct electroporation of schizont-infected RBCs (left panel), or by spontaneous DNA uptake in plasmid-loaded RBCs (right panel). The graphs show averages $\pm$ SEMs from three independent experiments in which luciferase measurements were obtained 48 h after transformation by schizont electroporation or after 72 h of culture for spontaneous DNA uptake from plasmid-loaded RBCs. Mock: control transformation experiments performed in parallel without plasmid; no parasites: control experiments with plasmid and uninfected RBCs only; LUs: luminescence units.

Fig. 3. Images of representative stages from tightly synchronized *P. knowlesi* cultures
 Parasitized RBCs observed by light microscopy (Giemsa stain). Developing schizonts are observed as soon as 18 h after RBC invasion by synchronized merozoites. Nearly mature and mature schizonts are detected 21 h after invasion and can be observed until burst and merozoite release 24 h after RBC invasion. Scale bar: 5 μ m

Fig. 4. Parasitemia and luciferase measurements of *P. knowlesi* pD-PfCam-luc transformants under WR99210 selection

A Parasitemias of morphologically-intact transformants obtained by direct electroporation of schizont-infected RBCs with pD-PfCam-luc or by growth in pD-PfCam-luc-loaded RBCs. Selection of the drug-resistant parasites by 1 nM WR99210 was initiated 48 h after schizont electroporation or 72 h after the first addition of plasmid-loaded RBCs for spontaneous transformation (grey arrows). **B** Luminescence measurements from samples of the transformant cultures. For each measurement, an 80 μ L volume containing $\sim 8 \times 10^7$ RBCs was removed from the culture and used for luciferase assay. Individual data points represent averages $\pm$ standard errors of the mean from three independent experiments of schizont electroporation and from two independent experiments of spontaneous plasmid uptake.

Table 1

Stage distributions and parasite numbers at the electroporation timepoints

Experiment ID*	Rings	Trophozoites	Developing schizonts	Nearly mature and mature schizonts	Gametocytes	Parasitemia	Luciferase signal (LUs) [‡]
SS#1 18 h	1	6	4	1	0	1.20%	3,620 ± 126
SS#1 21 h	0	1	5	5	0	1.10%	1,465,131 ± 581,935
SS#1 24 h	7	2	3	3	0	1.50%	526,487 ± 21,436
SS#2 18 h	0	18	9	0	0	2.70%	1,322 ± 4
SS#2 21 h	1	8	15	7	0	3.10%	161,190 ± 4,941
SS#2 24 h	0	3	13	16	0	3.20%	4,055,306 ± 221,242

* Schizonts were harvested for electroporation 18 h, 21 h and 24 h after reinvasion. Counts represent numbers of the different stages per 1,000 RBCs. Between 1×10^7 and 3×10^7 schizonts in a 100 μ L volume of pRBCs were used for each electroporation.

[‡] Luciferase measurements are from *P. knowlesi* schizonts harvested two days after direct electroporation (means $\pm$ standard deviations from two technical replicates; Table S4). LUs: Luminescence units.