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Generation of *Plasmodium yoelii* malaria parasite carrying double fluorescence reporters in gametocytes

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ABSTRACT

Male and female gametocytes are the infectious forms critical for malaria transmission and targets of intervention. Gametocytes are generally produced in relatively small numbers, and it has been difficult to obtain pure male and female gametocytes for various studies. Male and female gametocytes expressing unique fluorescence reporters have been generated for both *Plasmodium falciparum* and *Plasmodium berghei* parasites, which allows isolation of large numbers of pure male and female gametocytes and has greatly contributed to our understanding of gametocyte biology. To establish *Plasmodium yoelii* as another model for studying gametocytogenesis, here we generate a parasite line with male and female gametocytes expressing GFP or mCherry reporter, respectively, using CRISPR/Cas9-mediated gene editing method. We first inserted genes encoding intact fluorescence proteins downstream of parasite coding region of *ccp2* and *Dhc1* genes, respectively, generating the knockin parasites producing *ccp2::mCherry* (female) and *Dhc1::gfp* (male) gametocytes. We next obtained a parasite clone carrying double-fluorescent reporters by genetically crossing the *ccp2::mCherry* and *Dhc1::gfp* lines. The resulting double-labeled DFsc7 parasite displays normal development during the whole life cycle and expresses the fluorescence proteins in male and female gametocyte separately. This parasite strain provides a new platform for facilitating studies of gametocyte biology and malaria transmission.

1. Introduction

Sexual reproduction is an obligate step for malaria parasite transmission. In the vertebrate host, a parasite undergoes asexual intra-erythrocytic propagation. After cycles of asexual replication, a small number of asexual blood stages commit to sexual development, leading to male and female gametocytes that are the only forms capable of infecting mosquitoes. Upon ingestion by a mosquito, a single male gametocyte divides to form eight flagellated male gametes, while a single female gametocyte gives rise to a single immotile female gamete that is fertilized by a flagellated male gamete, forming a diploid zygote that develops into an ookinete.

Rodent malaria parasites *Plasmodium berghei* and *Plasmodium yoelii* have been widely used as models to study malaria pathology and parasite biology. These species have many biological characteristics similar to those of human malaria parasites during parasite development in vertebrate and mosquito hosts. To study sexual development, transgenic *P. falciparum* and *P. berghei* parasites expressing two fluorescence protein reporters mutual exclusively in male and female gametocytes have been generated [1,2]. The generation of fluorescence-labeled male and female gametocytes makes it possible to isolate large numbers of male and female gametocytes for various studies such as identification of sex specific genes and gender specific transcriptome and proteome [3–7].

Our laboratory uses *P. yoelii* as a model to study parasite development, particularly the development of gametocytes and mosquito stages. So far, transgenic parasites expressing two fluorescence reporters mutual-exclusively in male and female gametocytes are not available for *P. yoelii*. Here we report the generation of a marker-free *P. yoelii* 17XNL parasite line (DFsc7) expressing two fluorescence protein reporters in female and male gametocytes using CRISPR/Cas9-mediated editing method we developed previously [8,9], This parasite displays normal progression during the whole life cycle and expresses both mCherry and GFP reporters mutual-exclusively in female or male gametocyte. The generation of this DFsc7 parasite line allows efficient isolation of male or female gametocytes *via* FACS sorting. The parasite will be a useful tool for studying biology of *P. yoelii* gametocytes.

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2. Materials and methods

2.1. Animal use and ethics statement

All genetic modifications were performed in the *P. yoelii* 17XNL strain. The parasite was propagated in ICR mice (female, 5–6 weeks old) purchased from the Animal Care Center, Xiamen University. All mouse experiments were performed in accordance with approved protocols (XMULAC20140004) by the Committee for the Care and Use of Laboratory Animals at the School of Life Sciences, Xiamen University.

2.2. Plasmid construction, parasite transfection, and genotyping of modified parasites

To generate pYCm-based plasmid vectors for tagging gene *ccp2* (Gene ID PY17X_1323300 in PlasmoDB) with *mCherry* and for gene PY17X_0418900 with *gfp*, respectively, we amplified a C-terminal 600 to 700 bp of the coding region as the left arm and a 600 to 700 bp of the 3′ untranslational region (UTR) following the translation stop codon as the right arm using the primers in Table S1. The left and right arms were inserted into the restriction sites of the pYCm plasmid. The sgRNA sequences were designed to target sites close to either the 3′ UTR region or the C-terminal part of the coding region of the target genes. The procedures for parasite transfection, pyrimethamine (pyr) selection, and cloning were as described previously [8,9]. Briefly, purified parasites were electroporated with 5 μg purified circular plasmid DNA, immediately injected *i.v.* into a naïve mouse, and subjected to selection with pyr provided in drinking water at a concentration of 6 mg/L from day 2 after transfection. Pyr resistant parasites usually appear 5–6 days after drug selection. Parasite genomic DNAs from infected mouse blood samples were isolated and used for PCR genotyping. Correct 5′ and 3′ integrations were confirmed by PCR using two pairs of primers in Table S1.

2.3. Gametocyte induction and purification

Gametocyte induction and purification was performed using a modified protocol from [10,11]. Briefly, mice were treated by intraperitoneal injection of phenylhydrazine (PHZ, 80 μg/g mouse body weight) for hemolysis to enhance gametocytogenesis. Three days post treatment, mice were infected with 2.0×10^6 parasites by tail vein injection. Peaks of gametocytemia were usually observed three days post infection. Mouse blood containing more than 5% gametocytemia were collected and loaded for centrifugation on a Nycodenz cushion (27.6% w/v Nycodenz in 5.0 mM Tris−HCl, 3.0 mM KCl, 0.3 mM EDTA). Gametocytes were harvested from the interphase and washed three times with gametocyte maintenance buffer (137 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 20 mM glucose, 20 mM HEPES, 4 mM $NaHCO_3$) for further use.

2.4. Western blotting

Proteins extracted from purified gametocytes were separated on 4.5% SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore). The blot was incubated with blocking buffer (PBS with 3% bovine serum albumin) and then incubated with rabbit anti-mCherry (1:1,000, Abcam, ab167453) or rabbit anti-GFP (1:1,000, Cell Signaling Tech, #2956). Antiserum against BiP from our lab was used as a control. HRP-conjugated goat anti-rabbit antibody (1:5000, Sigma) was incubated with PVDF membranes for ECL detection.

2.5. In vitro ookinete culture

Ookinetes were prepared in *in vitro* culture according to the procedure [12]. Briefly, 200 μL of mouse blood containing gametocytes was mixed with 1 mL ookinete culture medium and incubated at 22 °C to allow gametogenesis, fertilization, and ookinete differentiation. The zygotes, retort, and mature ookinetes were taken from culture at 2, 6, and 12 h, respectively.

2.6. Mosquito infection and parasites quantification

Infected mouse containing more than 5% gametocytemia was anaesthetized and fed on 40–45 female *Anopheles stephensi* mosquitoes for 20 min. Twenty mosquitoes were dissected at day 7 post blood feeding and stained with 0.1% mercurochrome for midgut oocyst counting. Twenty mosquitoes were dissected at day 14 to collect salivary glands for sporozoite counting.

2.7. Expression of fluorescence proteins in recombinant parasites

Asexual blood stages and gametocytes from the infected mice, zygotes, retorts, and mature ookinetes from *in vitro culture*, day 7 midgut oocysts and day 14 salivary gland sporozoites from the infected mosquitoes were analyzed. For imaging living parasite, cells of different stages were washed twice with PBS before staining with Hoechst 33342 and observed under the fluorescence microscope. For imaging the fixed-cells, immunofluorescence assay (IFA) was performed as described previously [9]. Rabbit anti-mCherry (1:1,000, Abcam, ab167453), rabbit anti-GFP (1:100, Cell Signaling Tech, #2956), and mouse anti-α-Tubulin (1:1000, Sigma-Aldrich, T6199) were used as primary antibodies. P28 was labeled with rabbit anti-P28 antiserum (1:1,000, our lab). Goat anti-rabbit or anti-mouse antibodies conjugated with Alexa Fluor 488 or 555 were used as secondary antibodies. All images were captured and processed using identical settings in the Zeiss LSM 780 laser scanning confocal microscopy with a 100/1.49-numerical-aperture (NA) oil objective. Similar results were obtained in at least three independent experiments.

2.8. Flow cytometry cell analysis and cell sorting

The periphery blood was collected from mice carrying gametocytes after induction by PHZ. After two washes with PBS, the cells were suspended in PBS with Hoechst 33342 for nuclei staining and applied for Flow cytometry analysis using Fortessa (BD). Parasites infected RBCs (iRBC) were first gated out using positive signal of 405 nm. The GFP-positive and mCherry-positive cells were separated using 488 nm and 561 nm wavelength respectively. For the cell sorting experiments, the gametocytes of DFsc7 strain were first enriched by centrifugation on a 48% Nycodenz cushion according the protocol described above. The enriched gametocytes were then applied for cell sorting using MoFlo Astrios EQS (Beckman Coulter). Male and female gametocytes were sorted through the signal intensity of GFP and mCherry, respectively. After sorting, the cell populations were applied for FACS analysis for the cell purity. All the data were processed by FlowJo software.

2.9. Parasite genetic cross

Similar amount (1.0×10^6 parasites) of the asexual blood stage parasites of two recombinant lines were mixed to infect a PHZ-treated mouse through *i.v.* injection. Three days post infection, mice with high gametocytemia were prepared for mosquito infection. At day 14 post mosquito infection, a naïve ICR mouse was infected with salivary gland sporozoites through mosquito bite. Clones of blood stage parasites were obtained using limiting dilution, and genomic DNAs of cloned parasites were isolated for PCR genotyping.

3. Results and discussions

To obtain a *P. yoelii* parasite line expressing male or female gametocyte-specific fluorescence protein reporter, we inserted genes encoding mCherry and GFP downstream of two endogenous genes

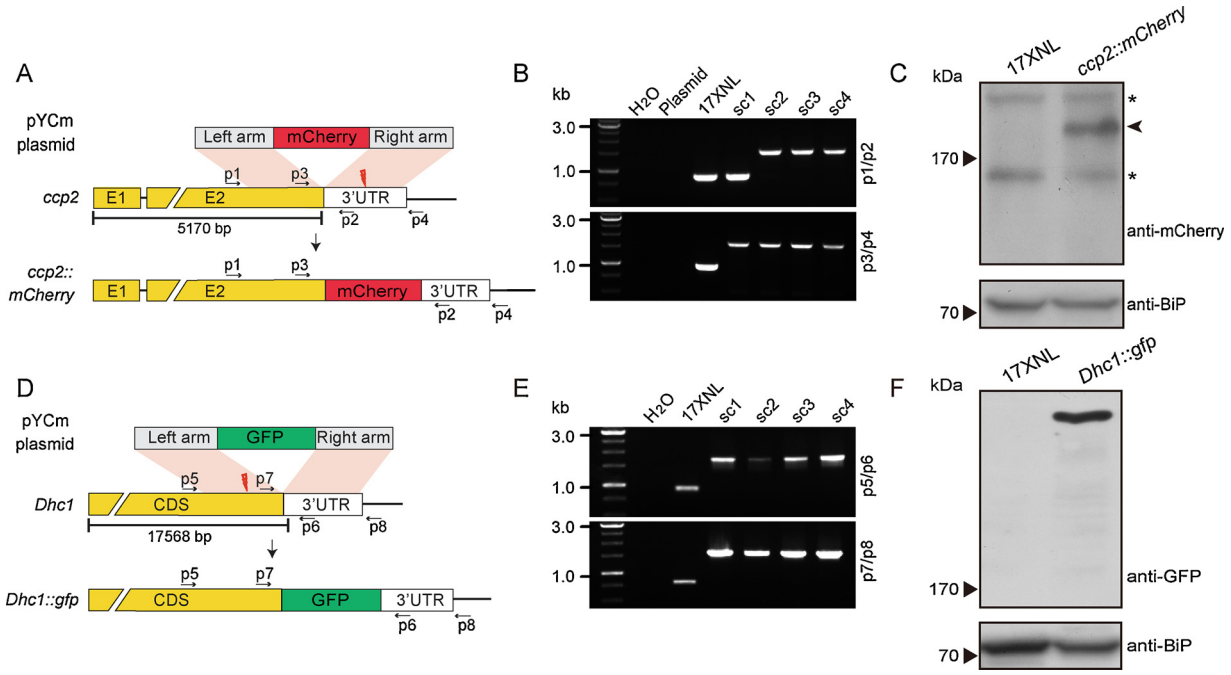

Fig. 1. Generation of *Plasmodium yoelii* 17XNL parasites with PY17X_1323300 (*ccp2*) tagged with *mCherry* and PY17X_0418900 (*Dhc1*) tagged with *gfp*. A) Diagram of CRISPR/cas9 plasmid pYCm construct for tagging *mCherry* at the C-terminal of the *ccp2* gene. "p" indicates the positions of PCR primers for genotyping analysis. The red thunderbolt indicates sgRNA recognition site. B) PCR detection of the *mCherry* integration in parasite clones. Primers p1/p2 and p3/p4 were used for detecting the 5′ and 3′ integrations, respectively. Sc1-sc4 are the genomic DNA of four individual parasite clones. C) Western blotting analysis of mCherry-tagged CCP2 protein in cell lysates of the purified gametocytes. Arrow indicates the band of CCP2::mCherry fusing protein. Star indicates the unspecific signal from the anti-mCherry antibody. Anti-BiP antibody was used as the loading control. D) Diagram of pYCm construct for tagging *gfp* at the C-terminal of the *Dhc1* gene. E) PCR detection of the *gfp* integration in parasite clones. Primers p5/p6 and p7/p8 were used for detecting the 5′ and 3′ integrations, respectively. Sc1-sc4 are the genomic DNA of four parasite clones. F) Western blotting analysis of GFP-tagged Dhc1 protein in cell lysates of the purified gametocytes.

(PY17X_1323300 and PY17X_0418900) separately. The gene of PY17X_1323300 encodes a 1636 amino acids (aa) LCCL domain-containing protein, CCP2. The orthologue of CCP2 has been shown to be critical for parasite development and transmission in mosquitoes for both *Plasmodium falciparum* and *Plasmodium berghei* [13,14]. The PY17X_0418900 gene encodes a putative dynein heavy chain protein (5855 aa, designated as *Dhc1* in this study) with unknown function in *Plasmodium*. The promoters of these two genes were reported to drive the expression of exogenous reporter proteins in male and female gametocytes of *P. berghei* respectively [1]. Using CRISPR/Cas9 methods described previously [8], we constructed a pYCm plasmid containing an intact gene encoding mCherry fluorescence protein flanked by 618 bp of the 3′ coding region and 601 bp of the 3′ untranslated region of *ccp2* gene as left and right homologous templates, respectively (Fig. 1A). After transfection, drug selection, and parasite cloning, we obtained four parasite clones (*ccp2::mCherry*, sc1 to sc4) from ten parasite-injected mice. Three clones had correct integrations of both homologous templates into the *ccp2* locus by double cross-over recombination that was confirmed by genotyping PCR analyses (Fig. 1B). Using the same method, we constructed another pYCm plasmid containing an intact GFP coding sequence flanked by left and right homologous sequences (675 bp of the 3′ coding region and 666 bp of the 3′ untranslated region) from the *Dhc1* gene (Fig. 1D). We again obtained four parasite clones with endogenous *Dhc1* tagged with GFP at C-terminal (*Dhc1::gfp*, sc1 to sc4) (Fig. 1E). Immunoblot analysis of the fused CCP2::mCherry protein extracted from purified gametocytes detected an about 210-kDa product in the *ccp2::mCherry* line (Fig. 1C), close to the expected size of the protein. In the *Dhc1::gfp* line, a specific band indicating Dhc1::GFP protein was detected (Fig. 1F). It is impossible to determine the accurate size of protein because the predicted molecular weight of Dhc1::GFP is 718-kDa. However, gene deletion of *Dhc1* in this *Dhc1::gfp* line confirmed the protein identity (data not shown). These results confirm corrected integrations of fluorescence protein tags into the targeted genomic locus.

Immunofluorescence assay (IFA) showed that the female gametocytes, which were indicated negative in α-Tubulin expression, express CCP2::mCherry protein in the cytoplasm of the *ccp2::mCherry* line (Fig. 2A, left panel). Furthermore, P28 protein, the protein marker for activated female gamete, zygote and ookinete, expresses and localizes to the periphery of all the mCherry-positive cells two hours post activation (Fig. 2A, right panel). These data indicate the female gametocyte specific expression of the mCherry-fused CCP2 protein. In the contrast, co-staining gametocytes of the *Dhc1::gfp* line with α-Tubulin and GFP antibodies showed male gametocyte-specific cytosol expression of Dhc1::GFP protein (Fig. 2B, left panels). Consistently, only GFP-positive gametocytes, but not GFP-negative parasites, display exflagellation with clear individual α-Tubulin labeled axonemes 15 min. post activation (Fig. 2B, right panels). In addition, the cell populations of either male (Dhc1::GFP-positive) or female (CCP2::mCherry-positive) gametocytes could be clearly detected using fluorescence-activated cell sorting (FACS) analysis (Fig. 2C). Together, these results show that the tagged *Dhc1* and *ccp2* proteins can be expressed properly in the male and female gametocytes, respectively. It is worth a note that treating mice with PHZ to enhance gametocytogenesis *in vivo* resulted in autofluorescence of a small subset of non-infected mouse red blood cells, which is observed in both FACS and fluorescence microscopy analysis (Figs. 2C and S1).

Next, we investigate stage expression of the tagged proteins during parasite life cycle. Using fluorescence microscopy, we detected the expression of CCP2::mCherry protein at gametocytes, zygotes, developing retorts and mature ookinetes (Fig. 3, left panel), but not at asexual blood stages, mosquito midgut oocysts and salivary gland sporozoites of the *ccp2::mCherry* parasite. Interestingly, CCP2::mCherry is expressed in the crystalloid organelle in ookinetes (Fig. 3). Crystalloids are derived from female gametocyte and function as a reservoir for protein required for sporozoite development within the oocyst [15].

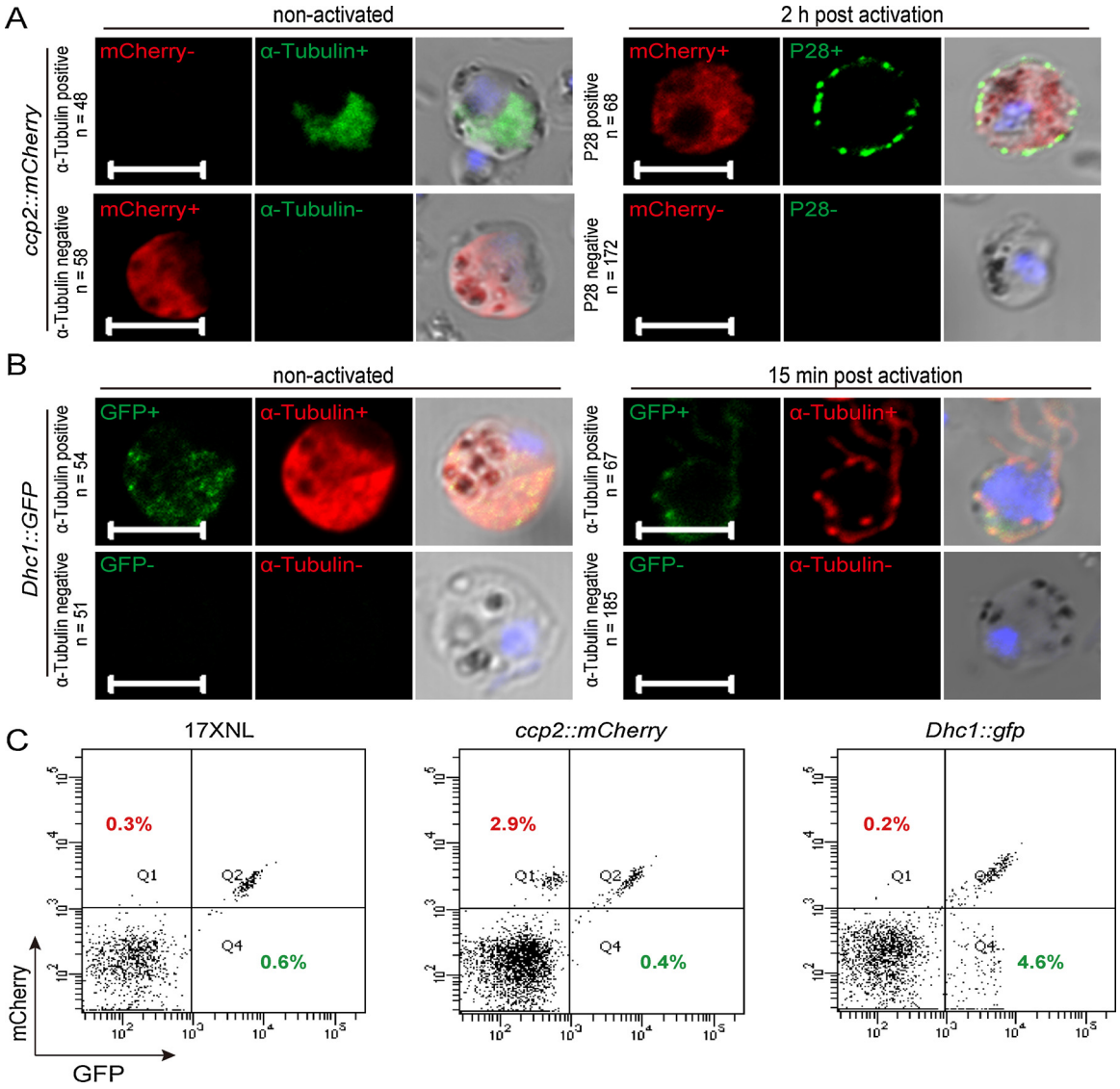

Fig. 2. Detection of tagged protein expression in male and female gametocytes. A) IFA for expression of mCherry, α-Tubulin, and P28 proteins in purified gametocytes of the *ccp2::mCherry* strain. α-Tubulin is a marker indicating male gametocytes, while P28 is expressed in activated female gametes, zygotes, and ookinetes. Nuclei were stained with Hoechst 33342 (blue). (n) is the number of cells analyzed in each group. Bar = 5 μm. The data are representative of three independent experiments. B) IFA for expression of mCherry and α-Tubulin proteins in purified gametocytes of the *Dhc1::gfp* line. (n) is the number of cells analyzed in each group. Bar = 5 μm. The data are representative of three independent experiments. C) Flow cytometry analysis of fluorescent cells of the *ccp2::mCherry* and *Dhc1::gfp* lines. Mouse blood stage parasites containing gametocytes were stained with Hoechst and the Hoechst-positive parasites were gated out for further mCherry and GFP expression analysis. The numbers are the percentages of female (red) and male (green) gametocytes compared with the total parasites. Note, there is auto-fluorescence signal from a small subset of non-infected mouse red blood cells after treatment with phenylhydrazine. For more information, see the Fig S1. The data are representative of two independent experiments.

These data suggest possible functions of CCP2 during parasite sporogony in the mosquito, in agreement with the phenotypes observed in gene disruption mutants of *P. falciparum* and *P. berghei* [13,14]. The expression of Dhc1::GFP protein was observed only in the gametocyte during life cycle of the *Dhc1::gfp* line (Fig. 3, right panel).

To obtain a parasite expressing both mCherry and GFP proteins in male and female gametocyte, respectively, we genetically crossed the *ccp2::mCherry* and *Dhc1::gfp* lines by co-infecting *Anopheles stephensi* mosquitoes with both parasite lines *via* natural bite (Fig. 4A). After 14 days development in the mosquitoes, the salivary gland sporozoites were used to infect the naïve mouse *via* mosquito bite to establish asexual blood stage infection (Fig. 4A). Seven clones were obtained after limiting dilution cloning, with a single clone carrying double integrated fluorescent markers (DFsc7 for double fluorescence single clone #7) (Fig. 4B). We compared the parasite development and differentiation during life cycle of the wildtype 17XNL, *ccp2::mCherry*, *Dhc1::gfp*, and DFsc7 parasites. All of the parasite strains displayed similar level of asexual proliferation and gametocytes formation in mouse blood (Fig. 4C, D), day 7 midgut oocysts and day 14 salivary gland sporozoites formation in the mosquito (Fig. 4E, F), and sporozoite infectivity to mouse (data not shown), indicating that tagging the endogenous CCP2 or Dhc1 protein with fluorescence protein has no effect on the parasite development during the life cycle. Importantly, the fluorescent signals of CCP2::mCherry and Dhc1::GFP proteins were clearly observed *via* fluorescence microscopy in the female and male cells, respectively, of the purified DFsc7 gametocytes (Fig. 4G). In addition, both the male and female cell populations could be easily differentiated using FACS (Fig. 4H). Furthermore, we successfully isolated highly purified female and male gametocytes (97% purity for mCherry-positive cells and 98% purity for GFP-positive cells) from the

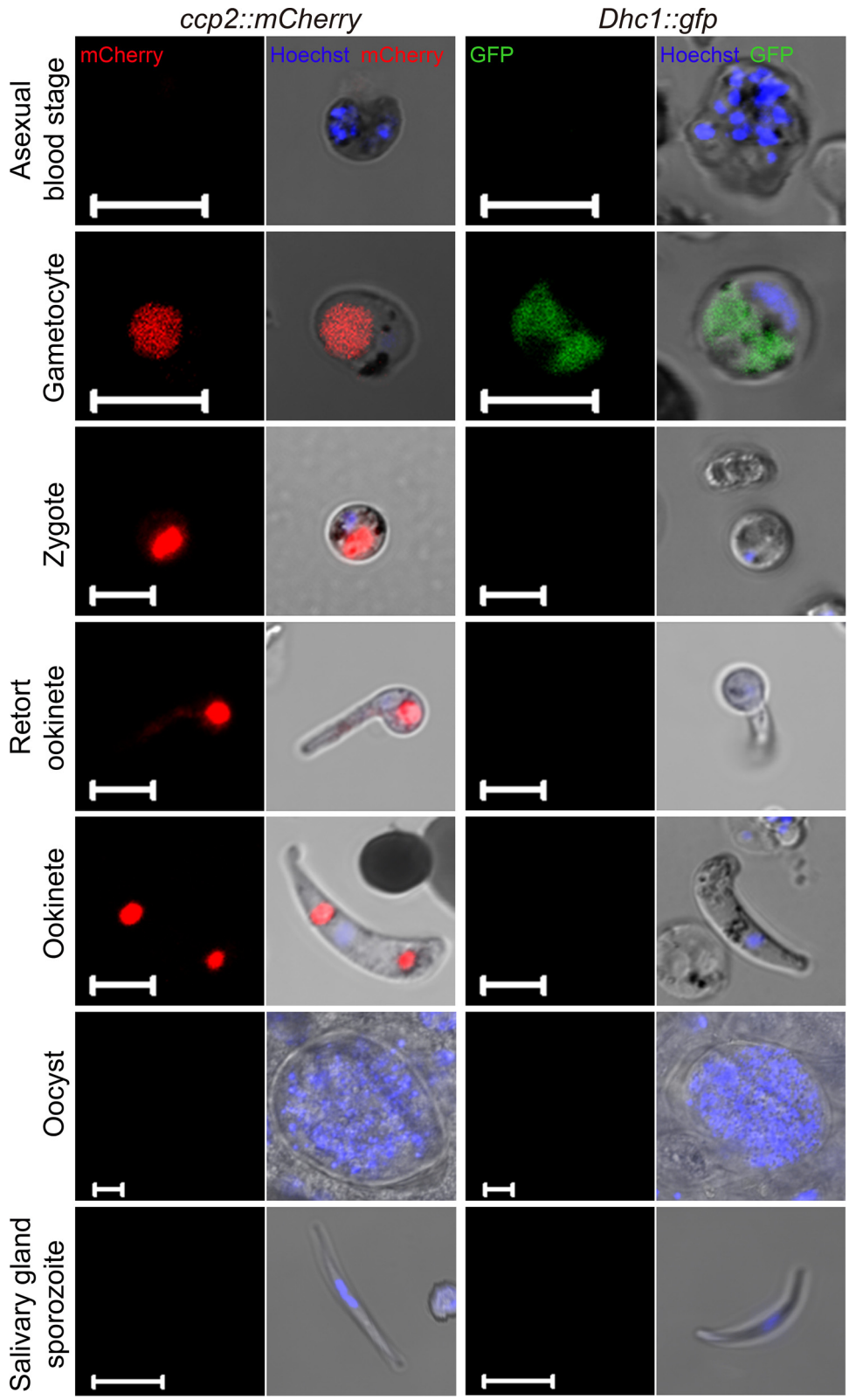

Fig. 3. Live cell imaging of mCherry and GFP protein expression during different life cycle stages of the *ccp2::mCherry* and *Dhc1::gfp* lines. Asexual blood stages and gametocytes from the infected mice, zygote, retort, and mature ookinete from *in vitro culture*, day 7 midgut oocyst and day 14 salivary gland sporozoite from the infected mosquitoes were analyzed using fluorescence microscope. The nuclei are stained with Hoechst (blue). Bar = 5 μm. The data are representative of two independent experiments.

gametocyte mixtures of the DFsc7 strain using FACS sorting (Fig. 4I).

During gametocyte activation, three-round genome DNA replication occurs specifically in male gametocytes, while activated female gametocytes keep haploid. By staining the parasite cell nuclei of the DFsc7 strain with Hoechst 33342 and FACS analysis, we could quantitatively compare the genome DNA content change of different cell

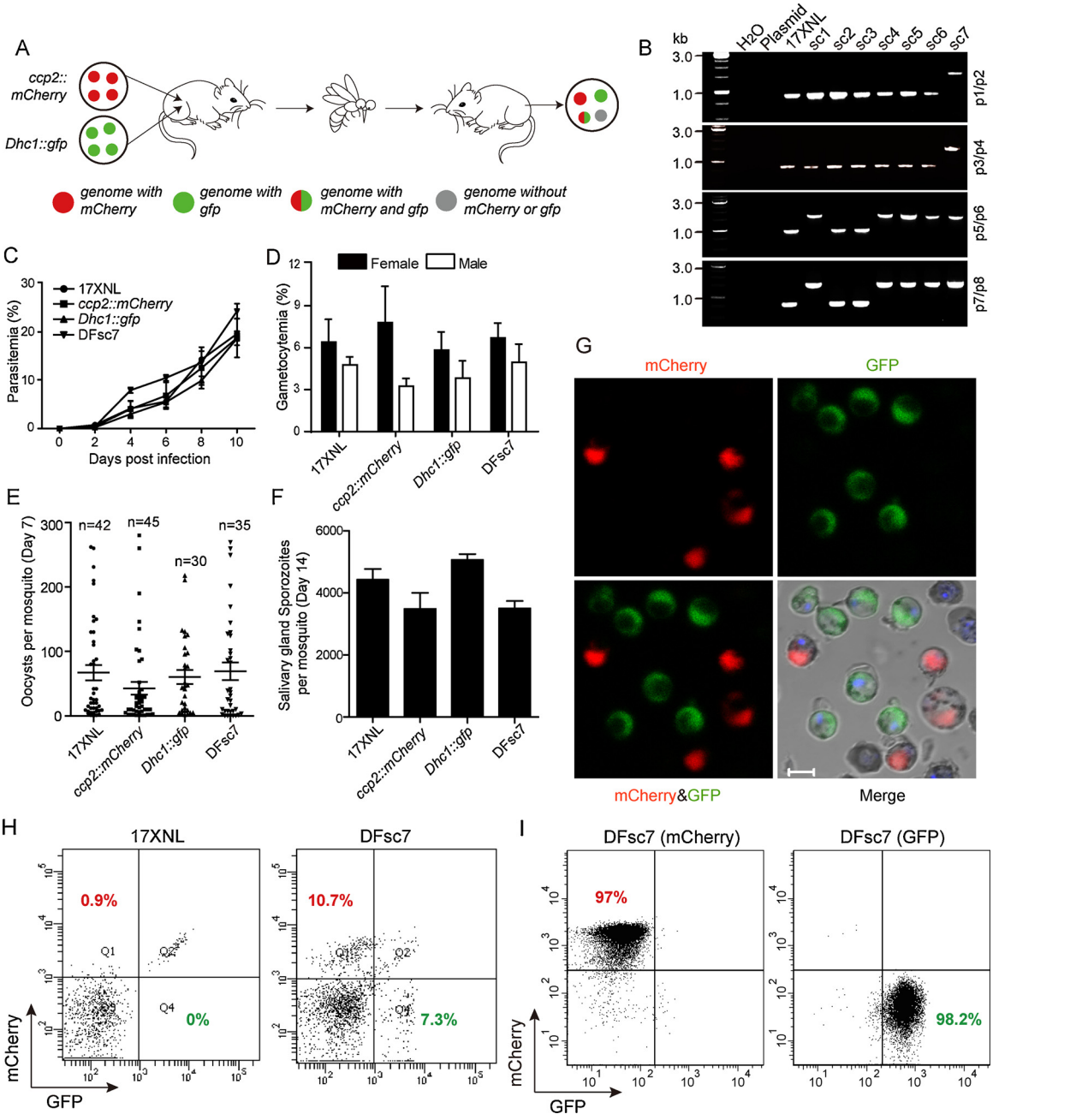

Fig. 4. Generation and characterization of the DFsc7 parasite expressing both fluorescent reporters in male and female gametocytes. A) Schematic for generation of the parasite expressing fluorescent reporters *via* genetic cross. A mouse was infected with both *ccp2::mCherry* and *Dhc1::gfp* lines and fed to mosquitoes by natural bite after detection of gametocytes in the blood. After 14 days development, the mosquitoes carrying salivary gland sporozoites were allow to feed on a naïve mouse to establish parasite blood stage infection. B) PCR detection of recombinant parasite clones containing both modified *ccp2* and *Dhc1* locus. Primers used were indicated in Fig. 1A and D. C) Proliferation of asexual blood stages in mice for the wildtype 17XNL, *ccp2::mCherry*, *Dhc1::gfp*, and the double reporter strain DFsc7. The results were means from three mice in each group. D) Gametocytes formation of the parasite lines. Female and male gametocytes were counted microscopically from mouse blood smear after Giemsa stain at day 3 post infection. E) Oocyst counts in the mosquitoes at day 7 post blood feeding. n is the number of mosquitoes dissected in each group. F) Salivary gland sporozoite counts in the mosquitoes at day 14 post blood feeding. The data are a representative of three independent experiments. G) Fluorescence microscopy of both mCherry and GFP protein expression in purified DFsc7 gametocytes. Bar = 5 μm. H) Flow cytometry analysis of fluorescent DFsc7 strain. Blood stage parasites containing gametocytes were stained with Hoechst and the Hoechst-positive parasites were gated out for further mCherry and GFP expression analysis. The data are representative of three independent experiments. I) Isolation and analysis of the female or male gametocytes by FACS sorting.

populations, including GFP population (male gametocytes), mCherry population (female gametocytes), and double-negative population (asexual blood stage, ABS) (Fig. 5). As we expected, about five-fold increase in DNA content was observed in only GFP population, but not in mCherry population after gametocyte activation of the DFsc7 strain (Fig. 5).

In summary, we report the generation of P. yoelii parasite lines (*ccp2::mCherry* and *Dhc1::gfp*) expressing individual mCherry and GFP reporter in female and male gametocytes, respectively. Furthermore, we crossed *ccp2::mCherry* and *Dhc1::gfp* lines to generate a parasite line carrying double-fluorescence reporters in both gender gametocytes. The resulted DFsc7 line displays normal progression during the whole life cycle and expresses two fluorescence proteins in male or female gametocyte separately. Importantly, both the male and female gametocyte

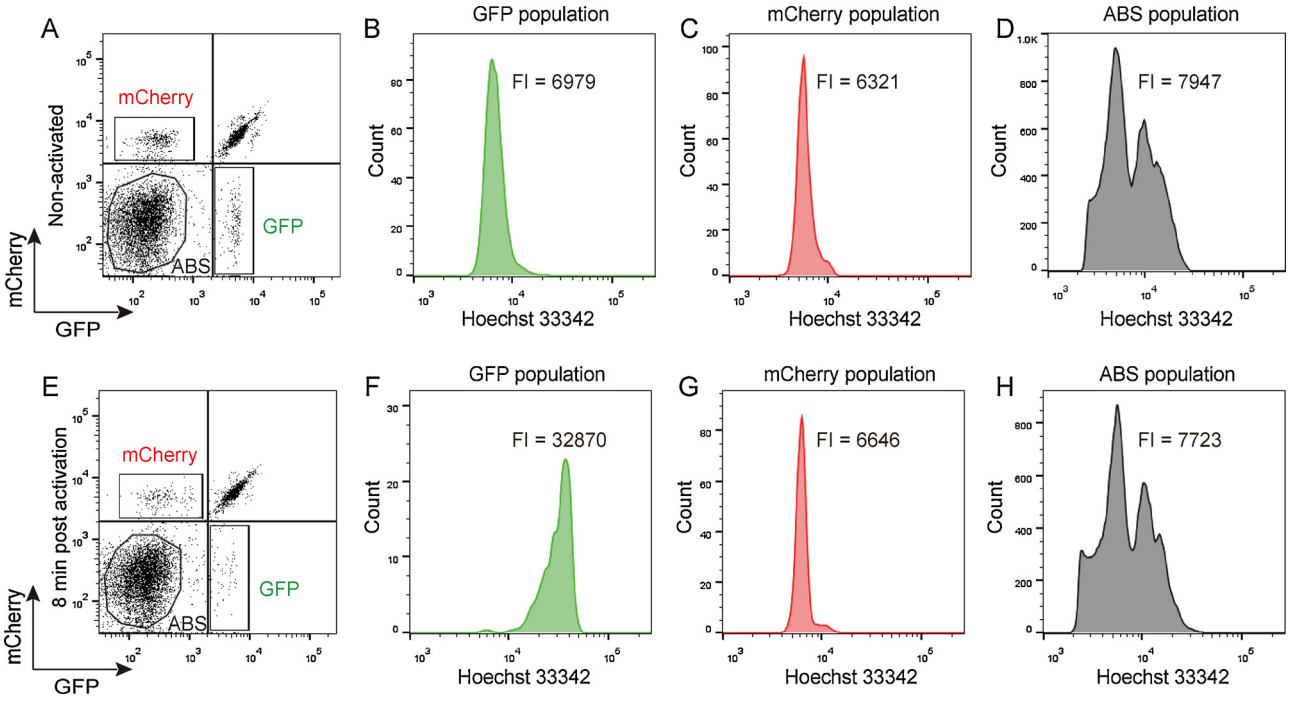

Fig. 5. Gametocyte DNA content analyses of DFsc7 strain after activation. A) Flow cytometry analysis of non-activated gametocytes of DFsc7 strain. Asexual blood stage (ABS) parasites and gametocytes were first gated out *via* staining positively with Hoechst 33342. The male gametocytes (GFP^+), female gametocytes ($mCherry^+$), and ABS parasites (double negative) could be differentiated. B–D) DNA content of cell populations, including GFP^+ (B), $mCherry^+$ (C), and double negative (D) from Fig. 5A. FI represents the mean fluorescence intensity of cells stained by Hoechst 33342. E) Flow cytometry analysis of gametocytes of DFsc7 strain at 8 min post activation. F–H) DNA content of cell populations from Fig. 5E.

populations could be easily differentiated using both fluorescence microscopy and FACS analysis. We also applied negative selection targeting *hdhfr-yfcu* selection cassette in the pYCm described previously [8] to remove the plasmid episome in the DFsc7 line. This selection marker-free reporter parasite is suitable for further genetic modifications and will be a useful platform for studying *P. yoelii* gametocyte and other sexual stages.

Acknowledgments

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