Supporting Information to article

"Multicolor bioluminescence boosts malaria research: quantitative dual-color assay and single-cell imaging in *Plasmodium falciparum* parasites"

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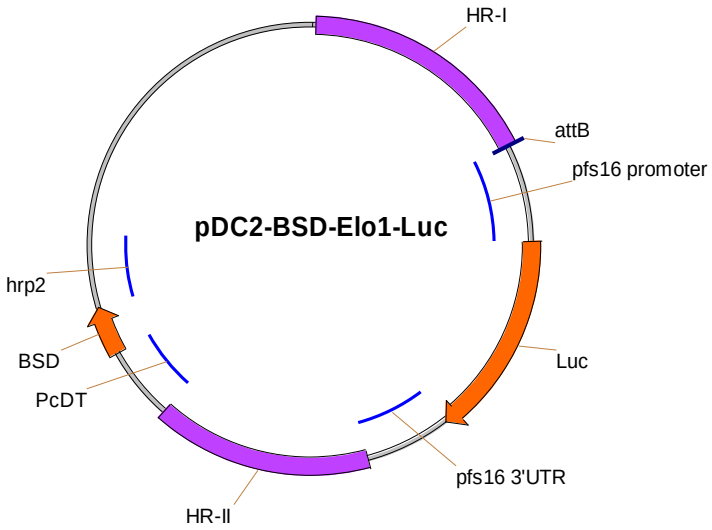
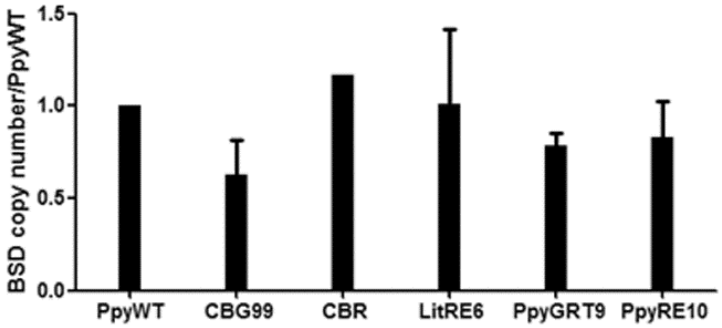
Supplementary Figure S-1

Representative map of the plasmids containing the luciferase reporter genes under the expression control of the *P. falciparum* gametocyte-specific gene *pfs16*.



Representative luciferase coding sequence (Luc) flanked by promoter and 3' untranslated region (3'UTR) of gene *pfs16*. Homology regions (HR) I and II of gene *pfelo1*. Coding sequence of the blasticidine S-deaminase (BSD) gene flanked by promoter of the *P. chabaudi* dihydrofolate reductase-thymidylate synthase gene (PcDT) and the 3'UTR of the *P. falciparum* histidine rich protein 2 gene (*hrp2*)

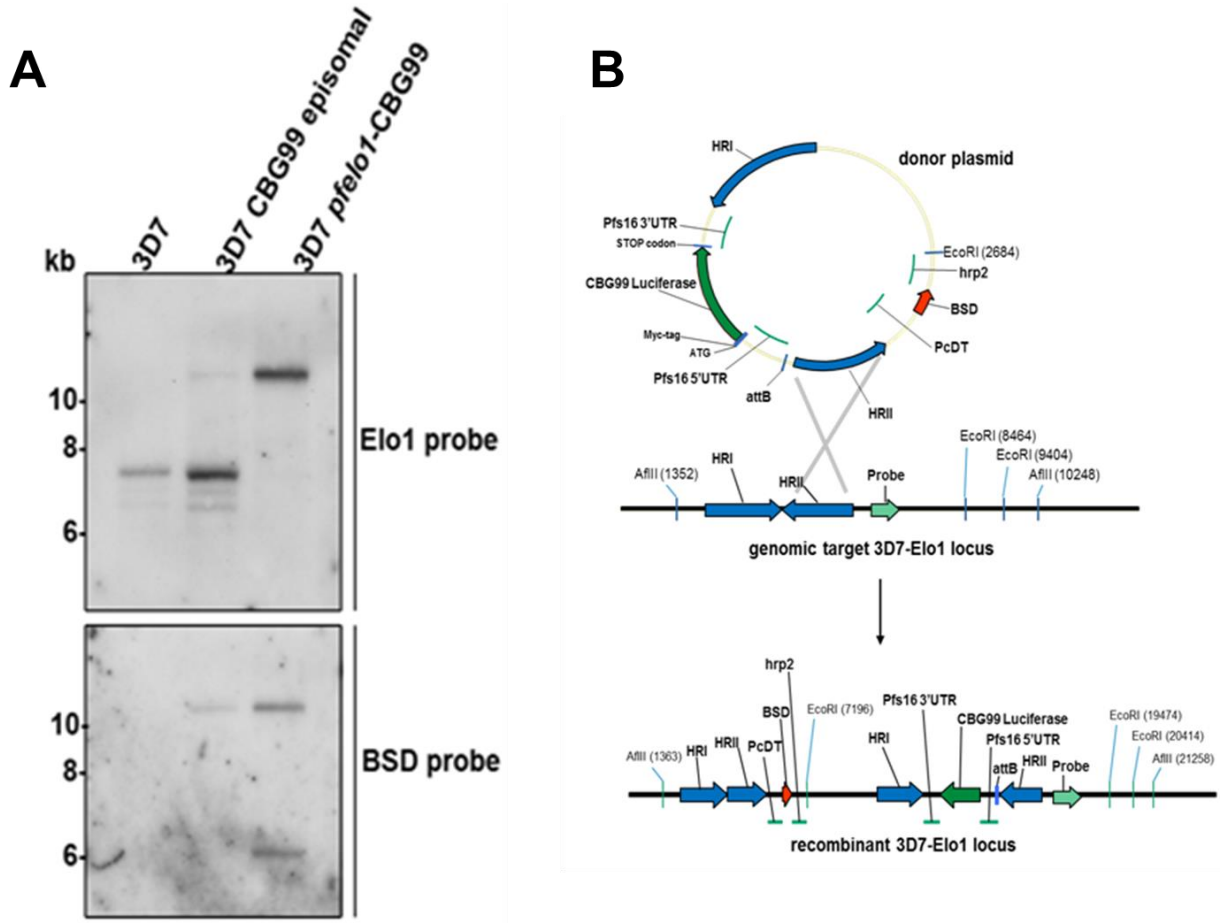
Supplementary Figure S-2. Relative copy number of the six luciferase expression plasmids in the *P. falciparum* transgenic lines.



SYBR-Green Real-time PCR experiments were performed as described in Materials and Methods amplifying the plasmid *bsd* and the parasite *pfeba175* gene sequences to determine plasmid copy number per parasite genome. Results of two independent experiments are expressed as fold over the PpyWT plasmid copy number (mean ± SD).

Supplementary Figure S-3

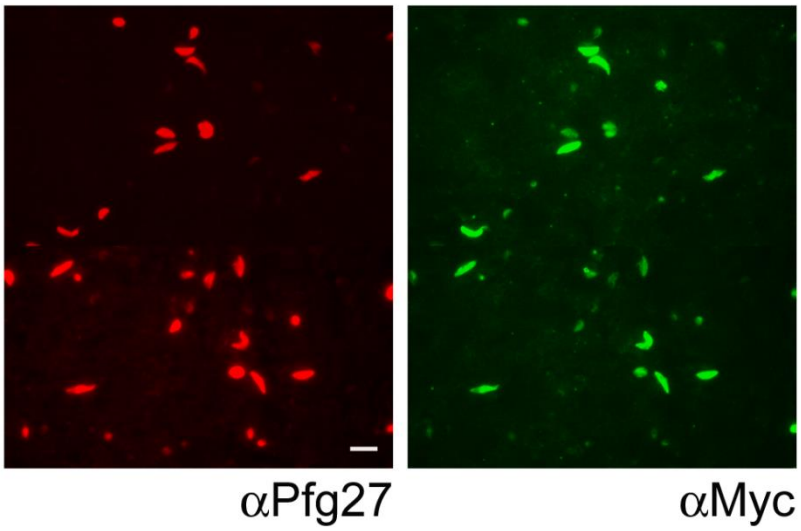
Production and characterization of the 3D7*elo1-pfs16*-CBG99 parasite line



A) Southern blot analysis of genomic DNA extracted from parasites of the parental line 3D7, of the 3D7 derived line episomally maintaining the *pfs16*-CBG99 plasmid and of the parasite line after single crossover plasmid integration. Genomic DNAs were digested with AflII and EcoRI. **B**) Diagram of the pfs16-CBG99 integration plasmid, of the target *pfelo1* locus and of the resulting modified locus.

Supplementary Figure S-4

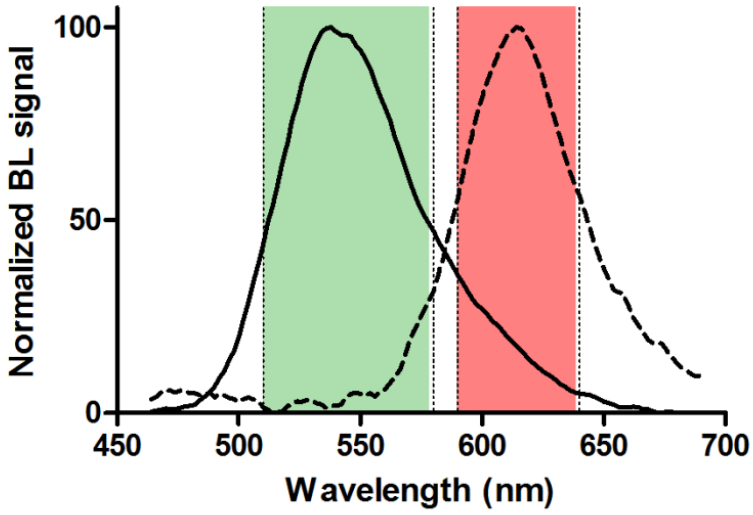
Immunofluorescence analysis of 3D7*elo1-pfs16*-CBG99 gametocytes.



Immunofluorescence analysis of 3D7*elo1-pfs16*-CBG99 gametocytes with antibodies specific for the Myc tag sequence fused to the CBG99 luciferase and for the gametocyte-specific protein Pfg27. Magnification bar is 10μm.

Supplementary Figure S-5

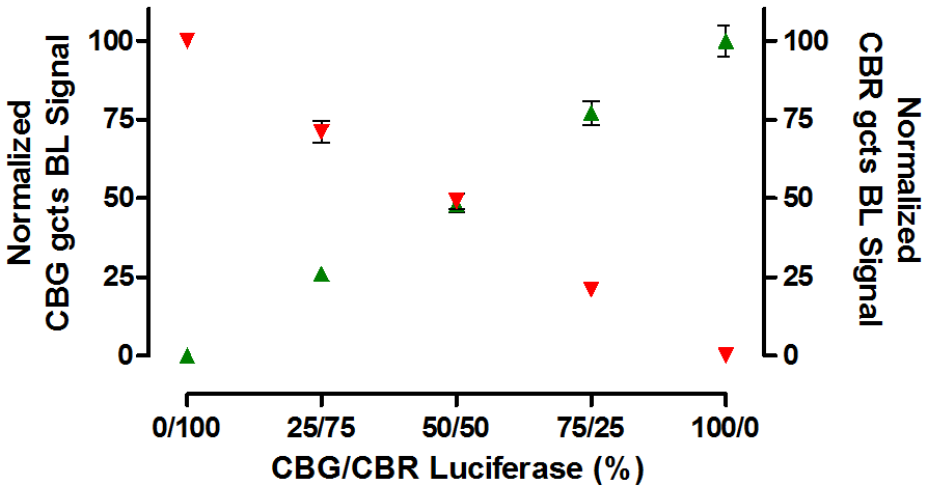
Bioluminescence emission spectra of the CBG99 (solid line) and CBR (dashed line) luciferases.



Wavelengths intervals of the green F545 (510-580 nm) and red F615 (590-640 nm) bandpass filters used for the simultaneous acquisition of the two selected luciferases with Varioskan luminometer were highlighted. The F545 allows to acquire about the 65% of the CBG and only the 5% of the CBR luciferase emission, whereas the F615 filter detect about the 70% of the red luciferase and the 16% of the green one. Due to this significant overlap between BL emissions, the introduction of a spectral unmixing algorithm (such as Chroma-Luc™ calculator) is needed for quantitative and reliable luciferase activity determination.

Supplementary Figure S-6

Spectral unmixing of CBG99- and CBR-luciferase expressing gametocytes.



CBG99 and CBR expressing gametocytes were mixed in different proportions corresponding to the indicated percentages of CBG/CBR luciferase activities. BL acquisitions were performed with Varioskan luminometer using the F545 and F615 filters. Raw BL emissions were elaborated with the ChromaLuc™ calculator spreadsheet to extract the corrected BL emissions, i.e. the contribution to the total light output of the green and red light emitting gametocytes. The relative CBG99/CBR combinations were chosen to mimic effects of compounds active on both stages or active mainly on one stage, leading to inhibition thresholds of 50% or 75%, typically used in hit compound identification.

Supplementary Experimental Section

Plasmid construction. The multistep procedure to obtain pCR2.1 vectors carrying myc-tagged luciferase expression cassettes equipped with Bbx1 attB sites under the expression control of the *pfs16* regulatory regions was as follows. First, attB-site adaptor, flanked by HindIII-SacI restriction site, was obtained by annealing oligonucleotides #1-attBsite-dir (all oligonucleotides used in this work are listed in Supplementary Table S1) and #2-attBsite-rev , digested and inserted into the HindII-SacI digested pCR2.1, producing pCR2.1-attB plasmid. Upstream *pfs16* regulatory regions were PCR amplified with primer pairs #3-pfs16-5'UTR-dir and #4-pfs16-5'UTR-rev for PpyWT, PpyRE10, PpyGRTS, and LitRE6, and primers #3-pfs16-5'UTR-dir and #5-pfs16-5'UTR-rev2 for CBG99 and CBR, and cloned into SacI-BamHI digested pCR2.1-attB after SacI-BamHI digestion, producing pCR2.1-attB-5'pfs16A and pCR2.1-attB- 5'*pfs16*B. The above different reverse primers were needed to introduce a myc-tag sequence in frame with downstream luciferases to be cloned. Downstream *pfs16* regulatory regions were PCR amplified with primers #6-pfs16-3'UTR-dir and #7-pfs16-3'UTR-rev and cloned into pCR2.1-attB-5'*pfs16* after XbaI-ApaI digestion, producing pCR2.1-attB 5'-3'*pfs16*A and pCR2.1-attB 5'-3'pfs16B. PpyWT, PpyRE10, and PpyGRTS, from pGEX expression plasmid were inserted into pCR2.1-attB-5' 3'pfs16A via BamHI-NotI cloning; LitRE6, from pCMV plasmid was inserted into pCR2.1-attB-5' 3'*pfs16*A via BamHI-XbaI cloning. CBG99 and CBR were obtained from pCBG99-basic and and pCBR-basic vectors (Promega) and cloned into pCR2.1-attB-5' 3'*pfs16*B via NcoI-XbaI.

The procedure to produce constructs able to mediate the chromosomal integration of the luciferase cassettes was as follows. First, a unique HindIII site in the pDC2 backbone site was disrupted by HindIII digestion and a Klenow fill-in reaction. An attB site was inserted by overlapping PCR between pfelo1 gene homology regions (HRs) as follows. As above, a HindIII site in pfelo1 HR-I was mutated by amplifying HRI using primers #8-pfelo1HR-I-dir and #9-pfelo1-HR-I-rev1, ligating it into pGEM-T plasmid (Promega). The resulting plasmid was linearised by HindIII digestion,

filled-in with Klenow polymerase, re-ligated and used as template for PCR amplification with above primer #8-pfelo1HR-I-dir and #10-pfelo1-HR-I-rev2. The latter contained, in 5' to 3' direction, stop codon, HindIII and attB sites. HR-II PCR amplification was performed with primers #11-pfelo1-HR-II-dir and #12-pfelo1-HR-II-rev. The former contained, 5' to 3', attB and HindIII sites. HR-I and HR-II PCR products were gel purified and mixed together in a PCR mix lacking primers and subjected to 5 cycles of amplification to generate a full-length HR-I-HindIII-attB-HindIII -HR-II template. Then, above primers #8-pfelo1HR-I-dir and #12-pfelo1-HR-II-rev were added to get the final PCR product, which was digested with ApaI-BamHI, gel purified and inserted into AvrII-BamH I digested pDC22-(mutHindIII) vector, producing the pfelo1-attB donor plasmid. Digestion of the latter plasmid with HindIII released the attB site which was replaced by the HindIII-HindIII attB-containing luciferase cassette from the pCR2.1 vectors.

Plasmid copy number determination by SYBR-Green Real-time PCR. The *bsd* selectable marker (primers #13-*bsd*-fw and #14-*bsd*-rev) was used along with the *pfeba175* gene (primers #15-*pfeba175*-fw and #16-*pfeba175*-rev) as a control for genome numbers. Genomic DNA was extracted from asexual cultures of episomal transfectants expressing the different luciferases alongside with parental 3D7 parasites using the Bioline Blood DNA kit. DNA (50ng in 2µl) was added to a 13µl PCR mix containing 3.7µl water, 7.5µl PCR Sybrgreen Master Mix and 0.9 µl of each primer (final concentration 300nM). Real-time assays were performed using ABI Prism 7500 Real-time PCR System (Applied Biosystems, Foster City, CA) and 7500 Software v2.0.5. The PCR parameters were as follows: 20 sec at 95°C followed by 40 cycles of 95°C for 15 sec and 58°C for 30 sec. Fluorescent product was detected at the last step of each cycle. Plasmid copy numbers were determined against reference amplification titration curves of plasmid (10^2 to 10^7 copies) in 25ng of 3D7 genomic DNA for *bsd* and of 3D7 genomic DNA (1.25 to 100ng) in 10^7 plasmid copies for *eba175*. All samples and controls were run in triplicate, normalized as plasmid copy number/genome and expressed as fold variation compared to PpyWT luciferase.

Southern blot analysis. Genomic DNA from the different parasite lines was analyzed as follows. Genomic DNA samples, 3µg, were digested with AflII and EcoRI, electrophoresed on a 0.8% agarose gel and transferred onto a Nytran nylon membrane. Hybridization of the membrane was performed at 54°C with a 639 bp [^{32}P]-labeled *pfelo1* probe that was PCR amplified from 3D7 genomic DNA using primers #17*elo1*probe-fwd and #18*elo1*probe-rev. The membrane was stripped in hot 0.1% SDS and hybridized at 54°C with a 389 bp [^{32}P]-labeled *bsd* probe amplified by PCR with primer #19*bsd*-probe-fw and #20*bsd*-probe-rev from plasmid pDC2-elo1-CBG99.

Supplementary Table S-1

List and sequence of the oligonucleotide primers used in the work.

#	Name	Sequence (5'-3')	Sites
1	attB site dir	ggggaagcttCGGCTTGTCGACGACGGCGGTCTCCGTCGTCAGGATCATCgagctcgggg	HindIII-SacI
2	attB site rev	ccccgagctcGATGATCCTGACGACGGAGACCGCCGTCGTCGACAAGCCGaagcttcccc	HindIII-SacI
3	*pfs16* 5' UTR dir	ggggGAGCTCCTACTGTACTTTTTTTTGGAC	SacI
4	*pfs16* 5' UTR rev1	ggggGGATCCCCATGGTAGGTCTTCTTCTGATATTAGTTTTTGTTCCATGTTGAAGAAAGTATAAATAGAAAAATGGC	BamHI
5	*pfs16* 5' UTR rev2	ggggGGATCCCCATGGtTAGGTCTTCTTCTGATATTAGTTTTTGTTCCATGTTGAAGAAAGTATAAATAGAAAAATGGC	BamHI
6	*pfs16* 3' UTR dir	ggggTCTAGAGATGAAGGAGACGAAGGAGATG	XbaI
7	*pfs16* 3' UTR rev	gggggggcccaagcttTATTTAGAGGTGAGGACTATG	ApaI
8	*pfelo1* HR-I dir	ggggatccACATGAATAAACTATTCACCCC	BamHI
9	*pfelo1* HR-I rev1	TCCACACGTATATATCGGAGG	
10	*pfelo1* HR-I rev2	GATCCTGACGACGGAGACCGCCGTCGTCGAAAGCCGAAGCTTCTAtccacacgtatatatcggagg	
11	*pfelo1* HR-II dir	GTCGACGACGGCGGTCTCCGTCGTCAGGATCATCGCGGAAGCTTatttggtacagttggattatgg	
12	*pfelo1* HR-II rev	TCGGGCCCGATTGCTTTTTCATTTTTTCCCTC	ApaI
13	*bsd* fw	TTGTCTCAAGAAGAATCCAC	
14	*bsd* rev	TAGAGAGAGCTGCGCTGGCG	
15	*pfeba*175 fw	TGGATAACACCAGTGAAGAAACTACAG	
16	*pfeba175* rev	CAATATCTTCATATTCCTTAGTAAGCG	
17	*elo1*probe-fwd	ACAGGCCGAGAAAATAAAGGAGAATGGC	
18	*elo1*probe-rev	CGGCATCTTGTTCTTGTACCATACAATG	
19	19*bsd*-probe-fw	GCACCTTTGTCTCAAGAAGAATCCACCC	
20	19*bsd*-probe-rev	GCCCTCCCACACATAACCAGAGGGCAGC	