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Phenotypic Analysis of Rodent Malaria Parasite Asexual and Sexual Blood Stages and Mosquito Stages.
--Manuscript Draft--

1 **TITLE:**
2 Phenotypic Analysis of Rodent Malaria Parasite Asexual and Sexual Blood Stages and Mosquito
3 Stages
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25 Malaria, *Plasmodium berghei*, *Plasmodium yoelii*, *Anopheles*, blood stages, gametocytes,
26 exflagellation, ookinetes, oocysts, sporozoites, liver stages
27

28 **SUMMARY:**
29 Due to the striking similarities of the life cycle and biology of rodent malaria parasites to human
30 malaria parasites, rodent malaria models have become indispensable for malaria research.
31 Herein, we standardized some of the most important techniques used in the phenotypic analysis
32 of wild-type and transgenic rodent malaria species.
33

34 **ABSTRACT:**
35 Recent advances in genetics and systems biology technologies have promoted our understanding
36 of the biology of malaria parasites on the molecular level. However, effective malaria parasite
37 targets for vaccine and chemotherapy development are still limited. This is largely due to the
38 unavailability of relevant and practical *in vivo* infection models for human *Plasmodium* species,
39 most notably for *P. falciparum* and *P. vivax*. Therefore, rodent malaria species have been
40 extensively used as practical alternative *in vivo* models for malaria vaccine, drug targeting,
41 immune response, and functional characterization studies of conserved *Plasmodium spp.* genes.
42 Indeed, rodent malaria models have proven to be invaluable, especially for exploring mosquito
43 transmission and liver stage biology, and were indispensable for immunological studies.
44 However, there are discrepancies in the methods used to evaluate the phenotypes of transgenic

45 and wild-type asexual and sexual blood-stage parasites. Examples of these discrepancies are the
46 choice of an intravenous *vs.* intraperitoneal infection of rodents with blood-stage parasites and
47 the evaluation of male gamete exflagellation. Herein, we detail standardized experimental
48 methods to evaluate the phenotypes of asexual and sexual blood stages in transgenic parasites
49 expressing reporter-gene or wild-type rodent malaria parasite species. We also detail the
50 methods to evaluate the phenotypes of malaria parasite mosquito stages (gametes, ookinetes,
51 oocysts, and sporozoites) inside *Anopheles* mosquito vectors. These methods are detailed and
52 simplified here for the lethal and non-lethal strains of *P. berghei* and *P. yoelii* but can also be
53 applied with some adjustments to *P. chabaudi* and *P. vinckei* rodent malaria species.
54
55 **INTRODUCTION:**
56 Malaria parasites cause hundreds of millions of malaria infections in humans worldwide, with
57 more than 600,000 deaths every year[1]. Human infections are caused by five malaria parasite
58 species, namely *P. falciparum, P. vivax, P. ovale, P. malariae*, and *P. knowlesi*. Most clinical
59 malaria mortalities are caused by *P. falciparum* in sub-Saharan Africa[1]. Another human malaria
60 parasite species that causes extensive worldwide morbidities outside of sub-Saharan Africa is *P.
61 vivax*[2]. The other three species are all more geographically restricted and cause benign malaria
62 infections, except the lethal *P. knowlesi*[3]. The unavailability of relevant and practical non-human
63 *in vivo* models of infections has always been and still is an obstacle to malaria vaccine and drug
64 development. Earlier malaria drug targeting and metabolic studies have relied extensively on
65 avian malaria models like *P. gallinaceum* and *P. lophurae*, infecting chickens and ducks,
66 respectively[4]. Thereafter, rodent malaria species were gradually introduced in various vaccines
67 and drug targeting studies as *in vivo* models. Over the years, evidence of similarities of the biology
68 and host-parasite interactions of life cycle stages of rodent malaria models to human malaria
69 species have accumulated.
70
71 In particular, rodent malaria models were extremely important to explore and characterize the
72 biology of mosquito and pre-erythrocytic stages[5]. However, there are four rodent malaria species
73 (*P. berghei, P. yoelii, P. chabaudi*, and *P. vinckei*) that have different biological features, the most
74 notable of which are in the blood stages[6]. Rodent malaria species differ in the synchronicity of
75 blood stages, where blood stages of *P. chabaudi* and *P. vinckei* strains are mostly synchronous,
76 while the blood stages of *P. berghei* and *P. yoelii* are not[6,7]. Another notable difference is the self-
77 clearance of blood stages that occurs in some strains (*e.g., P. yoelii* 17X-NL, *P. berghei* NK65, and
78 *P. vinckei lentum*), whereas the blood infection of other strains of the same species could be
79 lethal if left untreated (*P. yoelii* 17X-L, *P. berghei* ANKA, and *P. chabaudi* AS). Moreover, *P. yoelii*
80 17X-NL strain and *P. berghei* ANKA strain preferentially invade reticulocytes[8-11], although these
81 features of *P. yoelii* and *P. berghei* strains are not a strict growth requirement[12-14]. Therefore,
82 mice are treated with phenylhydrazine prior to an infection with the blood stages of those
83 parasites to increase the parasitemia and gametocytemia needed for a mosquito infection for
84 the *P. berghei* ANKA strain and for *P. yoelii* 17X-NL[15-19].
85
86 Differences in mosquito stages development also exist among different rodent malaria species,
87 the most notable being the temperature and time required for optimal mosquito stages
88 development and the sporozoite length[5,6,20]. In pre-erythrocytic stages of rodent malaria species,

89 differences include the rodent species and strain that are most susceptible to infectious
90 sporozoite inoculation, the number of sporozoites needed for inoculation in a susceptible rodent
91 strain, the mammalian cell types needed for *in vitro* liver stage development assays, and the time
92 to complete liver stage development[5,21-30].

94 Despite these variabilities, rodent malaria parasites were the favorable models early on for the
95 application of reverse genetic approaches, because they were less time- and resource-consuming
96 with a high probability of success[31]. In fact, rodent malaria models were the best models, and in
97 many instances the only models, available for numerous years to functionally characterize genes
98 expressed in mosquito and liver stages.

100 In light of the popularity and amenability of reverse genetic approaches in rodent malaria models,
101 a number of different methodologies have been utilized to analyze the phenotypes of transgenic
102 parasite life cycle stages, especially blood stages. However, some of these methodologies are
103 inconsistent; for instance, comparing infections of blood-stage parasites following an IP injection
104 (which are possibly drained to the peritoneal lymph nodes and, from there, can enter the
105 bloodstream; therefore, the injected parasites do not end up equally in the bloodstream),
106 comparing the mosquito transmission of clones with a different number of serial blood-stage
107 transfers or G number (which could affect gametocytogenesis[32,33]), or comparing transgenic
108 parasites directly to naive wild-type (WT) parasites that were never subjected to electroporation
109 and positive drug selection and the various unstandardized evaluations of male gamete
110 exflagellation. Therefore, it is crucial to standardize protocols that are simple to follow for the
111 phenotypic analysis of any type of transgenic or WT rodent malaria parasites in the blood and in
112 the mosquito to accommodate for the biological variabilities of rodent malaria parasite species.

114 Herein, we report on a standardized, detailed experimental protocol for the phenotypic analysis
115 of the blood and mosquito life cycle stages of transgenic or wild-type *P. yoelii* and *P. berghei*
116 parasites. These protocols are also applicable to *P. chabaudi* and *P. vinckei* parasites.

118 **PROTOCOL:**
119 All animal experiments described here were conducted according to the approved protocols of
120 the Institutional Animal Care and Use Committee (IACUC) of Tulane University. All other
121 experimental protocols and the use of recombinant DNA were conducted according to the
122 approved protocols of the Institutional Biosafety Committee (IBC) of Tulane University.

124 **1. Infection of Mice with Blood-stage Parasites for Parasitemia Analysis and Mosquito Infection**
125 **Assays**

127 **1.1. Day -3: optional injection of phenylhydrazine into recipient mice**

129 1.1.1. Inject outbred SW or CD1 recipient mice (mice that will be used for mosquito infection and
130 mosquito infection assays) intraperitoneally (IP) with 100 µL of phenylhydrazine (50 mg of
131 phenylhydrazine in 5 mL of 1x phosphate-buffered saline (PBS)) using a 26-G needle syringe.

Note: For robust mosquito infections of *P. berghei* and *P. yoelii*, phenylhydrazine is used to induce reticulocytosis in the recipient mice, which increases the parasitemia and, subsequently, the percentage of gametocytes, and significantly increases the exflagellation of male gametes (**Figure 5**), which will lead to a better mosquito stages infection for both *P. berghei* and *P. yoelii* strains[15-18].

1.2. Day -2: injection of frozen parasite stocks into donor mice

1.2.1. Quickly thaw cryopreserved frozen stocks and immediately inject each donor mouse (typically two mice per genotype) IP with ~200 µL of stock using a 26-G needle syringe.

Note: No more than five mice are housed per cage within controlled vivarium settings.

1.2.2. Start checking parasitemia under a 100X microscope objective on Giemsa-stained thin blood smear after 24 h postinfection. Follow section 2.1 of the protocol for Giemsa-stained thin blood smears.

Note: Due to the inability to precisely count the surviving viable blood stages after quickly thawing and injecting cryopreserved infected blood, donor mice receive the cryopreserved blood first. The infected erythrocytes of the donor mouse can be precisely quantified and used subsequently.

1.3. Day 0: bleeding of the donor mice

1.3.1. Bleed the donor mice (see section 3.1) when their parasitemia is between 0.1% and 1%, which is usually obtained 2 - 3 d post-frozen-stock-infection in the case of *P. yoelii* 17X-NL and *P. berghei* ANKA strains.

1.3.2. Use the facial vein puncture (to get between 200 and 500 µL), or terminally bleed the animals by heart puncture (to get between 600 µL and 1.2 mL) of infected blood. Terminally bleed the mice by heart puncture as described in detail in section 3.1.

Note: Facial vein bleeding is not a routine method of obtaining blood as it is much more difficult to apply and is very distressful to mice, compared to terminal bleeding. The optimal parasitemia for the bleeding of donor mice is between 0.1% - 1% because, at this parasitemia range, there are very few gametocytes (which cannot replicate to produce asexual blood stages) and there are less double- or triple-infected erythrocytes (which makes the quantification less accurate).

1.4. Day 0: quantification of infected blood to prepare doses of infected erythrocytes by serial dilutions

1.4.1. Prepare serial dilutions of donor blood by adding 100 µL of blood to a microcentrifuge tube (dilution tube 1). Add 900 µL of RPMI medium to tube 1 and mix well by pipetting up and down with the same pipette tip, creating a 1:10 dilution.

177

178 1.4.2. Take 100 µL of the diluted blood from tube 1 and add it to a new microcentrifuge tube
179 (dilution tube 2). Add 900 µL of RPMI medium to tube 2 and mix well by pipetting up and down
180 with the same pipette tip, creating a 1:100 dilution.

181

182 1.4.3. Repeat steps 1.4.1 and 1.4.2 to make two more serial dilutions, creating 1:1,000 (dilution
183 tube 3) and 1:10,000 dilutions (dilution tube 4).

184

185 1.4.4. Add 12 µL of diluted blood from tube 3 and 12 µL of diluted blood from tube 4 to different
186 sides of a hemocytometer and determine the average number of erythrocytes on each side of
187 the hemocytometer. Multiply these numbers by 10. Multiply these numbers by the dilution
188 factor, 1,000 or 10,000, respectively, to determine the number of erythrocytes in 1 µL of each
189 dilution.

190

191 1.4.5. Divide the desired number of infected erythrocytes to be injected by the number of
192 erythrocytes in 1 µL of each dilution to determine the volume of diluted donor blood necessary
193 for injection. Choose the dilution with the most appropriate volume for injection, add the volume
194 to a new microcentrifuge tube, and complete it to 120 µL with RPMI medium.

195

196 **1.5. Day 0: intravenous injection of the recipient mice**

197

198 1.5.1. Place the recipient mice under a heat red lamp to dilate their veins for injection. Load the
199 parasite doses in a 27-G insulin syringe and place the recipient mouse in a restrainer.

200

201 1.5.2. Inject 1 million or 10,000 infected erythrocytes intravenously (IV) using the 27-G insulin
202 syringe in each recipient mouse for mosquito infection assays or for asexual and sexual blood-
203 stage growth assays, respectively. Continue to maintain the mice under standard housing
204 conditions.

205

206 Note: An IP injection is an indirect method of introducing parasites into the bloodstream, as
207 parasitized erythrocytes have to pass through the draining lymph nodes of the peritoneal cavity
208 to reach the blood. This makes an IP injection an indirect delivery route for injecting a precise
209 number of parasites into the bloodstream. Therefore, the IV route is preferred, as it delivers
210 accurate parasite doses directly into the bloodstream, as shown in **Figure 3** and described in the
211 **Representative Results**.

212

213 **2. Determination of the Blood-stage Parasite Load for Sexual and Asexual Stages**

214

215 Note: In this section, standardized phenotypic evaluation methods of malaria parasite blood
216 stages are listed. These methods are useful in the evaluation of novel antimalarial or vaccine
217 candidates or even gene knockout on sexual and asexual stages development in the same
218 experimental mice. Of note, *P. chabaudi* and *P. vinckei* are also very rational and important
219 alternative options for these types of assays, especially in drug screening.

220

221 **2.1. Determination of the parasitemia of asexual stages and male *vs.* female gametocytes by**
222 **Giemsa-stained thin blood smears for parasite growth assays**
223
224 2.1.1. Using a 26-G or 24-G needle, prick the mouse's tail and collect the blood droplet on a
225 microscope slide. Use the short edge of another microscope slide to quickly smear the blood
226 across the slide.
227
228 2.1.2. Fix the slide with 100% methanol for at least 1 min, only after the blood has completely
229 dried on the slide. Stain in 10% Giemsa for 10 - 15 min. Wash the slide with single- or double-
230 distilled water and allow it to dry.
231
232 2.1.3. Read the slide using a 100X objective and oil immersion under a light microscope. For an
233 accurate parasitemia measurement, check at least a total of 6,000 erythrocytes, which can be
234 obtained by counting at least 30 or 40 microscopic grids with an average number of 200 or 150
235 erythrocytes (RBCs) per grid, respectively. Count the total number of RBCs in the first and last
236 grids to determine the average number of red blood cells for all grids.
237
238 2.1.4. Count the total number of infected erythrocytes per grid. Male and female gametocytes
239 can be counted separately to determine gametocytemia but should also be included in the total
240 parasitemia count.
241
242 2.1.5. Determine the total parasitemia percentage by dividing the total number of parasitized
243 erythrocytes by the total number of RBCs observed. Multiply this number by 100 to determine
244 the parasitemia percentage.
245
246 2.1.6. Determine the gametocytemia by dividing the total number of gametocytes counted by
247 the total number of RBCs observed. Multiply this number by 100 to determine the
248 gametocytemia percentage per mouse.
249
250 Note: The only reliable method of differentiation between different asexual and sexual blood
251 stages is the use of a microscope to evaluate Giemsa-stained thin blood smears, as each of the
252 stages has different morphology, with the exception of immature gametocytes, which are mostly
253 indistinguishable from asexual blood stages.
254
255 **2.2. Determination of the blood-stage parasitemia by flow cytometry for fluorescent reporter**
256 **protein-expressing WT-like parasite strains**
257
258 2.2.1. Label two microcentrifuge tubes for each mouse sample, designating one tube for a 1:1,000
259 dilution and the other for a 1:2,000 dilution. Add 1.5 μL of 1x heparin to the 1:1,000 dilution tube.
260
261 2.2.2. Using a 26-G or 27-G needle, prick the mouse's tail and transfer 1.5 μL of blood to the tube
262 containing 1.5 μL of 1x heparin (the 1:1,000 dilution tube). Mix the blood and the 1x heparin
263 gently together by pipetting up and down.
264

265 2.2.3. Add 1,497 µL of RPMI medium to the microcentrifuge tube, and gently pipette up and down
266 to mix well.

268 2.2.4. Transfer 500 µL of the mixture from the 1:1,000 dilution tube to the 1:2,000 dilution tube.
269 Add 500 µL of RPMI medium to the 1:2,000 tube and gently mix by pipetting up and down.

271 2.2.5. Follow the manufacturer's appropriate start-up protocol for the flow cytometer. Run the
272 samples at a slow flow rate and set the detection for at least 20,000 events.

274 Note: There is an alternative practical assay to measure parasitemia *via* tricolor FACS sorting,
275 whether parasites express fluorescent markers or not[34]. However, the assay described here
276 offers an accurate measure of parasitemia in live parasites without the addition of any DNA-
277 binding dyes and the use of FACS sorting.

279 **2.3. Determination of the exflagellation rate of male gametes per microliter of infected mouse**
280 **blood**

282 2.3.1. Prepare a 1:10 dilution microcentrifuge tube with 40 µL of incomplete ookinete medium
283 (RPMI + sodium bicarbonate + hypoxanthine + xanthurenic acid) and 5 µL of 1x heparin.

285 2.3.2. Using a 26-G or 27-G needle, prick the mouse's tail and transfer 5 µL of blood (using a
286 micropipette) quickly to the tube containing the incomplete ookinete media + heparin. Mix gently
287 and load 12 µL of the 1:10 blood dilution tube onto a hemocytometer and leave it at room
288 temperature (20 - 24 °C).

290 2.3.3. Start counting the exflagellation events 9 - 10 min after preparing the 1:10 dilution. Use a
291 phase contrast light microscope with 40X magnification.

293 2.3.4. Multiply the average number of exflagellation events counted by 10 and then multiply by
294 the dilution factor (10) to determine the average number of exflagellation per microliter of
295 infected blood.

297 Note: This assay measures the number of male gamete exflagellation events in a 1-µL volume of
298 blood, which can be quantified using a hemocytometer. An incomplete ookinete medium[18] is
299 mixed with the blood to closely mimic the conditions during which exflagellation occurs inside
300 the mosquito midgut shortly after a blood meal.

302 **3. Isolation and Processing of Blood-stage Parasites from Infected Erythrocytes and Frozen**
303 **Stock Preparation**

305 **3.1. Terminal bleeding of infected rodents by heart puncture**

307 3.1.1. Prepare a 26-G needle syringe that contains approximately ≤ 20 µL of 1x heparin (200 units/
308 ml) to collect the blood of the donor mouse by heart puncture.

309

310 3.1.2. Use a slow flow of CO_2 to euthanize the mouse before bleeding. Alternatively, anesthetize
311 the mouse with isoflurane (which has to be maintained before and during the heart puncture).

312

313 3.1.3. Lay the mouse on its back and pin down its appendages. Make a small incision in the skin
314 near the base of the sternum by pulling up the skin with forceps and cutting it with scissors. Pull
315 the skin apart at the site of the incision to expose the diaphragm and the top of the abdominal
316 cavity.

317

318 3.1.4. Hold the base of the rib cage with forceps and cut through the diaphragm to enter the
319 thoracic cavity; be careful not to damage the heart or lungs. Pin back the rib cage to expose the
320 heart.

321

322 3.1.5. Gently puncture the heart and slowly pull up on the syringe plunger to collect ~1 mL of
323 blood and transfer it to a microcentrifuge tube.

324

325 **3.2. Preparation of frozen stocks from infected blood**

326

327 3.2.1. Bleed the mice (see section 3.1) with blood parasitemias that are between about 2% and
328 5%, with a significant amount of ring stages and not as many gametocytes.

329

330 3.2.2. Mix the desired portion of blood with freezing solution (10% glycerol in Alsever's solution)
331 in a 1:2 ratio and store in cryogenic vials. Freeze in liquid nitrogen.

332

333 **3.3. Isolation and purification of blood-stage parasites for the extraction of DNA, RNA, and**
334 **proteins**

335

336 3.3.1. Bleed mice (see section 3.1) with blood parasitemias that are higher than 0.5%.

337

338 3.3.2. Prepare a 10-mL syringe by removing the plunger and inserting a small cotton swab. Pack
339 down the cotton to the bottom of the syringe. Fill it with cellulose until the level of cellulose
340 reaches the 3-mL mark but does not exceed the 4-mL mark on the syringe.

341

342 3.3.3. Secure the syringe into a clip on a ring stand and place a waste collection tube below it.
343 Add 5 mL of PBS to the syringe and allow it to flow through into the waste tube.

344

345 3.3.4. Prepare frozen stocks if needed, using only 100 - 300 µL of blood. Add the rest of the
346 infected blood (400 - 700 µL) to the column. Collect flow through with a waste tube until the
347 droplets start to turn red. Once the droplets start to turn red, place a new 14-mL collection tube
348 under the syringe to collect the flow through.

349

350 3.3.5. Once the flow starts to slow, add PBS to the syringe and collect the flow through in the 15-
351 mL tube until a total volume of 14 mL has been reached. Collect the remaining flow through with
352 a waste tube.

353

354 3.3.6. Centrifuge the 14-mL tube with blood/PBS mixture for 8 min at 250 x g with no brakes.
355 Remove the supernatant and add 14 mL of chilled saponin (50 mg of saponin in 50 mL of PBS).
356 To dislodge the pellet, invert the tube multiple times and vortex if necessary.

357

358 3.3.7. Centrifuge the tube for 8 min at 1217 x g with no brakes. Remove the supernatant, leaving
359 about 0.5 mL of the solution above the pellet.

360

361 3.3.8. Resuspend the pellet in the solution using a micropipette and transfer it to a
362 microcentrifuge tube. Add 500 µL of PBS to the 14-mL tube to wash any residual parasites that
363 are left in the tube. Add this to the same microcentrifuge tube.

364

365 3.3.9. Centrifuge the tube for 2 min at 6010 x g. Remove the supernatant and resuspend 1 mL of
366 PBS. Centrifuge for 2 min at 9391 x g.

367

368 3.3.10. Proceed to extract genomic DNA, total RNA, and protein.

369

370 3.3.10.1. For **genomic DNA extraction**, remove the supernatant and resuspend the pellet in 200
371 µL of PBS, and then follow any suitable protocol of gDNA extraction.

372

373 3.3.10.2. For **total RNA extraction**, remove the supernatant, resuspend and vortex the pellet in
374 1 mL of any phenol- and guanidine isothiocyanate-based solution or any other suitable reagent
375 and, then, follow any suitable protocol of total RNA extraction.

376

377 3.3.10.3. For **total protein extraction**, remove the supernatant, resuspend the pellet in an
378 appropriate volume of 6x sodium dodecyl sulfate (SDS)-loading dye or any other suitable reagent
379 and, then, follow any suitable protocol of total protein processing.

380

381 **4. Mosquito Infection Assays**

382

383 Note: The mosquito is the primary host of the malaria parasites where sexual reproduction takes
384 place. The infection of mice to transmit malaria parasites to mosquitoes is conducted by an IV
385 injection of at least 1 million blood stages, followed by feeding an infected mouse (from each
386 genotype that displays the highest male gamete exflagellation rate) to *Anopheles* mosquitoes in
387 a cage at day 3 post-mouse-infection. The IV injection with 1 million blood-stage parasites in
388 phenylhydrazine-treated mice will ensure the development of male and female gametocyte at a
389 faster and higher rate. Mosquitoes infected with *P. yoelii* and *P. berghei* are incubated at 24 °C
390 and 20 - 22 °C, respectively, to allow for the best possible mosquito stages development[6].

391

392 **4.1. Mosquito feeding and determination of the number of ookinetes per mosquito**

393

394 4.1.1. Starve adult female *Anopheles stephensi* or *A. gambiae* mosquitoes (4 - 7 days old) for 8 -
395 12 h prior to feeding. Allow adult mosquitoes to feed for at least 15 min on the infected

anesthetized mice (injected with an appropriate dose of ketamine/xylazine) with the highest exflagellation rate (measured in section 2.3).

Note: Ketamine/xylazine working solution is prepared by the 1:5 dilution of the stock solution in saline and IP injecting 100 µL per mouse. For a 10-mL stock solution, 1 mL of xylazine (100 mg/mL) is added to 9 mL of ketamine (100 mg/mL).

4.1.2. Remove unfed female mosquitoes and male mosquitoes with a mouth aspirator.

4.1.3. At 18 - 20 h postfeeding, collect 20 - 30 mosquitos and place them in the freezer for no longer than 5 min to ensure death. Using a binocular dissection scope, dissect out 20 - 30 blood-filled midguts with two 26-G or 27-G needles or a needle and forceps in RPMI or PBS dissection medium and transfer the midguts to a microcentrifuge tube containing 200 µL of RPMI medium.

4.1.4. Centrifuge the collection tube for 1 min at 4,000 x g. Grind the pelleted midguts using a pestle. Repeat this step.

4.1.5. Transfer 50 µL from the tube of ground midguts to a new microcentrifuge tube. Add 200 µL of RPMI medium to create a 1:5 dilution.

4.1.6. Transfer 12 µL to a hemocytometer and incubate it at room temperature for 5 min to allow the contents to settle.

4.1.7. Determine the average number of ookinetes by using a light microscope with a phase contrast and 40X magnification.

4.1.8. Multiply the average number of mature ookinetes by 10 and then by the dilution factor of 5 to determine the average total number of ookinetes.

4.1.9. Divide the average number of ookinetes by the number of blood-fed mosquitoes dissected to determine the number of ookinetes per blood-fed mosquito.

4.2. Determination of the number of early oocysts for fluorescent reporter protein-expressing WT-like parasite strains

Note: The aim of this assay is to determine the number of live parasites expressing green fluorescent protein (GFP) that established a full infection of the mosquito midguts and formed early spherical oocysts. This assay determines if the ookinetes (their development estimated in the previous assay) completed their functions by the traversal through the mosquito midgut epithelial cells and the transformation to early oocysts on the basal lamina side of the midgut epithelia or not. This is another assay which makes great use of the parasites expressing GFP, as counting early oocysts at this stage will be nearly impossible without tedious immunostaining.

439 4.2.1. Dissect the midguts (see section 4.1) of 20 - 30 blood-fed mosquitoes, at day 3 or 4 post-
440 mosquito-feeding (pmf) for *P. yoelii* 17X-NL, and at day 6 or 7 pmf for *P. berghei* ANKA, with two
441 26-G or 27-G needles or a needle and forceps in RPMI or PBS dissection medium.
442
443 4.2.2. Hold the thorax in place with one needle (or forceps) and carefully pull away the abdomen
444 with the other needle. The midgut should remain attached to the thorax after the removal of the
445 abdomen. Separate the midgut from the thorax using a needle.
446
447 4.2.3. Spread 40 - 50 μL of sterile dissection medium on the horizontal midline of the longer edge
448 of the glass slide. Transfer the midguts to this line, one at a time, during dissection.
449
450 4.2.4. Place a coverslip gently on the dissected midguts, and seal it with nail polish.
451
452 4.2.5. Using the 10X or 20X objective of the fluorescence microscope with the green fluorescence
453 filter, count the number of early oocysts on each midgut, in at least 20 midguts.
454
455 **4.3. Determination of the number of oocyst sporozoites per mosquito**
456
457 4.3.1. Dissect the midguts of at least 20 - 30 mosquitoes with two 26-G needles or a needle and
458 forceps in RPMI or PBS dissection medium and collect the dissected midguts in 200 μL of RPMI.
459
460 4.3.2. Hold the thorax in place with one needle (or forceps) and carefully cut, then pull away the
461 abdomen with the other needle. The midgut should remain attached to the thorax after the
462 removal of the abdomen. Cut the midgut away and transfer the midguts of all mosquitoes to the
463 collection tube by collecting them from the medium with a needle.
464
465 4.3.3. Centrifuge the collection tube for 1 min at 94 x *g* to settle the midguts to the bottom of the
466 collection tube and grind them with a pestle. Repeat this step.
467
468 4.3.4. Transfer 12 μL to a hemocytometer and incubate it at room temperature for 5 min to allow
469 its contents to settle.
470
471 4.3.5. Count the oocyst sporozoites using a 40X objective of a light microscope. Use 1:5 and/or
472 1:10 dilutions if mosquito debris prevents the precise counting of the sporozoites or if the
473 number of sporozoites is too high to count accurately.
474
475 4.3.6. Calculate the average total number of oocyst sporozoites by multiplying the average
476 number of sporozoites by 10 and, then, multiplying that number by the dilution factor and the
477 total volume (200 μL).
478
479 4.3.7. Divide the average total number of sporozoites by the number of mosquitoes dissected to
480 calculate the average number of oocyst sporozoites per mosquito.
481

482   Note: A common mistake for measuring the success or productivity of mosquito stages infection
483   is the counting of the number of oocysts at later stages of oocyst development. This is due to
484   some oocysts being vacuolated at later time points of oocyst development, and others fail to
485   develop sporozoites, even on the same midgut. The best and most reliable method to determine
486   the productivity of mosquito stages infection is the counting of the average number of midgut
487   oocyst sporozoites at days 10 - 12 post-mosquito-infection for *P. yoelii* and at days 12 - 14 post-
488   mosquito-infection for *P. berghei*.

490   **4.4. Determination of the number of salivary gland sporozoites per mosquito**

492   Note: The ultimate evaluation of the full completion of mosquito stages development is
493   accomplished by estimating the number of sporozoites that invaded the salivary gland, which are
494   the transmissible and infectious stages to vertebrates. The invasion of the salivary gland is
495   established after the completion of oocyst sporozoite development, egress into the hemolymph,
496   attachment to the basal lamina of the salivary gland acinar cells, and traversal of the acinar cells
497   to reach the salivary glands ducts[5]. Therefore, this assay is also an evaluation of the success of all
498   of these processes or not. Nonetheless, an estimation of hemolymph sporozoite numbers would
499   allow the differentiation between defects in the egress from oocysts and defects in the invasion
500   of salivary glands[35]. The purification of salivary gland sporozoites allows conducting multiple
501   functional assays on sporozoite motility and invasion phenotypes. The salivary gland sporozoites
502   can also be used for the *in vivo* infection of mice and in immunization studies[36,37]. Moreover, the
503   most reproducible stages available to establish an *in vitro* liver stage invasion or a development
504   assay is by the use of salivary gland sporozoites.

506   4.4.1. Dissect the salivary glands of 50 - 100 female mosquitoes (see section 4.1), at days 14 - 16
507   pmf for *P. yoelii* and at days 17 - 20 pmf for *P. berghei*, preferably with two 26-G or 27-G needles,
508   in RPMI or DMEM medium kept on ice and supplemented with 5% fetal bovine serum
509   (FBS)/bovine serum albumin (BSA) for *P. yoelii* sporozoites or 3% FBS/BSA for *P. berghei*
510   sporozoites. If the sporozoites are to be used in hepatoma *in vitro* assays, add 1%
511   penicillin/streptomycin to the dissection medium.

513   4.4.2. Separate the head of the mosquito from the thorax with the side of the needle without
514   applying any pressure to the thorax.

516   4.4.3. With the back of the beveled part of the needle, apply pressure to the anterior side of the
517   thorax. Collect the whitish material that oozes from the neck using a short glass Pasteur pipette
518   and transfer it to a microcentrifuge tube. Repeat this step for all mosquitos.

520   4.4.4. Centrifuge the collection tube for 1 min at 845 x *g* to pellet the thoracic tissue masses.
521   Transfer any medium that exceeds ~300 µL to another tube to use when washing the sporozoites.

523   4.4.5. Grind the thoracic tissue masses using a pestle. Centrifuge again at for 1 min at 845 x *g*
524   and regrind it with a pestle. Transfer all the contents to an Ozaki method tube after wetting the

525 tube first with 200 µL of RPMI medium[38].

526

527 Note: The Ozaki method tube is a 600-µL microcentrifuge tube perforated at its bottom by a 21-
528 G needle and stuffed with a small ball of fiberglass wool and placed in a 1.5- or 2-mL
529 microcentrifuge tube.

530

531 4.4.6. Centrifuge for 2 min at 845 x g and collect the flow through in a new tube. Wash the tube
532 with the excess collection medium with ≥ 300 µL of RPMI medium (volume equivalent to the first
533 flow through). Combine the flow through with the flow through from the previous step.

534

535 4.4.7. Count sporozoites using a hemocytometer under a light microscope set to Phase 2 contrast
536 and 40X magnification. If the number of the mosquitos dissected ≥ 40, dilute the salivary glands
537 to 1:5 or 1:10, depending on the expected infectivity yield (determined in earlier assays).

538

539 4.4.8. Multiply the average number of sporozoites by 10 and the dilution factor (if any). Multiply
540 by the total volume to determine the average number of salivary gland sporozoites. Divide by the
541 number of dissected female mosquitoes to determine the average salivary gland sporozoites per
542 mosquito.

543

544 Note: The problem with the dissection of the salivary glands is their small size and glassy
545 transparent appearance. Thus, it is very difficult to isolate the salivary glands and, therefore, they
546 are usually dissected out as a lump sum with other tissues from the frontal part of the thorax,
547 which is also important to protect the salivary glands from rupture during the collection.

548

549 **REPRESENTATIVE RESULTS:**
550 The success of applying reverse genetic tools and techniques to malaria parasites has
551 revolutionized the field of malaria research, with the ability to add, delete, or modify specific
552 genomic segments of several *Plasmodium* species[39]. Importantly, dispensable genomic loci have
553 been identified and used successfully to introduce fluorescence protein markers in rodent and
554 human malaria parasites by double homologous recombination, to ensure a stable expression in
555 all life cycle stages[40-42]. An example of these WT-like transgenic parasites is *Py230p(-)* parasites,
556 which have been generated in our lab, and showed no apparent defect in the development of
557 blood and mosquito life cycle stages[15-17]. These transgenic reporter parasites expressed eGFP,
558 under the control of the strong and constitutive promoter of *PyHSP70*, in blood stages (**Figure
559 1A**) ookinetes (**Figure 1B**), young oocysts (**Figure 1C**) on *Anopheles stephensi* midguts, and in
560 sporozoites isolated from the salivary glands of *Anopheles stephensi* females (**Figure 1D**). Thus,
561 the eGFP-expressing blood parasites made it much easier and less time-consuming to evaluate
562 blood-stage parasitemia using flow cytometry between different genotypes of transgenic
563 parasites or between drug-treated and -untreated in drug-targeting assays using transgenic
564 reporter parasites.

565

566 In order to confirm that there is no quantitative difference between the use of flow cytometry
567 and the more tedious parasitemia estimation by microscopy, the eGFP-expressing *Pyp230p(-)*
568 was used to estimate the percentage of parasitized erythrocytes by flow cytometry and by

569 Giemsa-stained thin blood smear in a group of Swiss Webster mice IV-infected with 10,000
570 parasitized erythrocytes. The flow cytometry parasitemia% values corresponded directly to the
571 estimated parasitemia% by monitoring Giemsa-stained thin blood smears, which were estimated
572 by two expert scientists (**Figure 2**). This represents a more accurate alternative to the tedious
573 and prone-to-human-error method of microscopy in the determination of the growth rate of
574 blood-stage parasites.
575
576 An important discrepancy associated with the infection of rodents with malaria parasites blood
577 stages is the choice of route of infection, with a strong preference in the literature for the IP
578 compared to IV route of infection, as it is less time-consuming. In order to compare these two
579 routes of infection, two groups of five BALB/c mice were infected with 1,000 eGFP-expressing
580 *Pyp230p(-)* parasitized erythrocytes per mouse, either through IV or IP routes. The parasitemia
581 was monitored daily using flow cytometry for a period of 4 days. A statistically significant
582 decrease in the IP-infected group parasitemia% compared to the IV-infected group was noted on
583 all days tested (**Figure 3**). This provides evidence that the IV infection route is a more
584 quantitatively accurate route of infection for assays with the malaria parasite blood stages.
585
586 Nonetheless, one limitation to the use of flow cytometry to evaluate blood-stage parasitemia is
587 the differentiation between sexual and asexual stages and between male and female
588 gametocytes. Therefore, the estimation of the percentages of each of the different asexual and
589 sexual stages (**Figure 4**) has to depend on a morphology evaluation of Giemsa-stained thin blood
590 smear. Despite the apparent different morphology of mature male and female gametocytes
591 (**Figure 4**), immature sexual stages are often indistinguishable from asexual stages.
592
593 One essential function of the male gametes upon emergence from male gametocyte in the
594 mosquito midgut is the male gamete exflagellation, which is a very critical step in the
595 transmission that must happen within a very short period of time. Variable methods used to
596 evaluate this in many different systems have been described. Herein, we show a standardized
597 method that can be repeated in any simple lab setting. We evaluated male gamete exflagellation
598 with or without the injection of phenylhydrazine into recipient mice (**Figure 5**). We could show
599 that the phenylhydrazine treatment significantly (four fold) increased the rate of male gamete
600 exflagellation, which in turn will increase the fertilization rate and the number of all subsequent
601 mosquito stages.
602
603 **FIGURE LEGENDS:**
604
605 **Figure 1: The development of *P. yoelii p230p(-)* parasites constitutively expressing eGFP in**
606 **blood and mosquito stages.** (**A**) Image of mixed blood-stage parasites (1,000X magnification). (**B**)
607 Image of ookinete (400X magnification). (**C**) Image of an *Anopheles stephensi* mosquito midgut
608 infected with day 4 pmf early oocysts of live *p230p(-)* parasites expressing eGFP (100X
609 magnification). (**D**) This panel shows a live image of a *P. yoelii p230p(-)* salivary gland sporozoite,
610 dissected out at day 15 pmf, expressing eGFP (400X magnification).
611

612 **Figure 2: Flow cytometry and microscopy evaluations of average blood-stage parasitemia of *P.***
613 ***yoelii p230p(-)* parasites are not significantly different.** A group of four Swiss Webster mice was
614 intravenously infected with 10,000 parasitized erythrocytes of *Pyp230p(-)* per mouse and the
615 average blood-stage parasitemias% were recorded daily for 7 days by flow cytometry and by the
616 microscopic evaluation of Giemsa-stained thin blood smears from a total of 20,000 and ~6,000
617 erythrocytes, respectively. The microscopic examination results shown are the average of two
618 readings by two expert scientists per slide, and the time for evaluating the parasitemia of each
619 slide was at least 10 minutes by each scientist. No significant differences could be detected on
620 any of the days shown here. The mean values for all parasite strains were analyzed with the two-
621 tailed *t*-test. The error bars represent the standard deviation.

622

623 **Figure 3: An intravenous injection of *P. yoelii p230p(-)* parasites yields significantly different**
624 **starting blood-stage parasitemia from an intraperitoneal injection.** Two groups of five BALB/c
625 mice were infected with 1,000 parasitized erythrocytes of *Pyp230p(-)* per mouse, either through
626 the intravenous or intraperitoneal route, and the average blood-stage parasitemias% were
627 recorded daily for 4 days by flow cytometry from a total of 20,000 erythrocytes. A statistically
628 significant reduction (denoted by an asterisk) of blood-stage parasitemia could be detected for
629 all days tested in the intraperitoneal route group compared to the intravenous route group. The
630 mean values for all parasite strains were analyzed with the two-tailed *t*-test. The error bars
631 represent the standard deviation.

632

633 **Figure 4: Morphology of *P. yoelii* gametocytes in a Giemsa-stained thin blood smear.** An image
634 of a Giemsa-stained thin blood smear (1,000X magnification) of a Swiss Webster mouse infected
635 with WT *P. yoelii* 17X-NL strain shows the typical blueish colored female gametocyte on the left
636 side (denoted by an arrow) and the pinkish colored male (denoted by an asterisk) gametocyte on
637 the right side of the image. The other stages shown are asexual blood stages.

638

639 **Figure 5: The effect of phenylhydrazine on male gamete exflagellation.** The effect of
640 phenylhydrazine injected in recipient mice 5 days prior to the male gamete exflagellation rate
641 estimation of *P. yoelii*. Phenylhydrazine significantly increases the rate of male gamete
642 exflagellation, which leads to a higher mosquito stages infection post-mosquito-feeding. The
643 mean values for all parasite strains were analyzed with the two-tailed *t*-test. The error bars
644 represent the standard deviation.

645

646 **DISCUSSION:**
647 In spite of the similarity in the general biology of their life cycle to that of human malaria
648 parasites, mouse malaria models also have many dissimilarities to human *Plasmodium* species
649 that would limit their use as reliable *in vivo* models. For instance, with the exception of live-
650 attenuated parasites as vaccines, all vaccine studies with subunit and DNA and other vaccines
651 gave excellent results in the mouse model, but in humans living in endemic areas, the results
652 were far from satisfactory.

653

654 Another problem is the difference of life cycle stage infectivity from one mouse strain to another,
655 and sometimes from one animal vendor to another for the same mouse strain. Moreover, the

656 main two rodent malaria species that are widely used as preferred *in vivo* malaria models, *P.*
657 *berghei* and *P. yoelii*, do not exhibit a synchronous blood-stage cycle, which is completely
658 different from any human malaria parasite. However, the benefits of using mouse malaria
659 parasites as *in vivo* models by far outweigh these dissimilarities, which can be overcome by more
660 in-depth analyses of the molecular drive of these limitations. In general, these limitations are
661 mostly displayed by the rodent malaria blood-stage parasites, but not as much for the other life
662 cycle stages of the malaria parasite.
663
664 Although blood stages are important for various vaccine, drug targeting, immunology, and
665 functional genomic studies, there is a scarcity of standardized methods and protocols that
666 concentrate on the phenotypical analysis and functional assays that involve rodent malaria
667 parasite blood-stage parasites, with more focus on mosquito transmission and transfection
668 protocols. Therefore, the methods in this article will help to provide standardized and simplified
669 protocols for studying the pathogenic stages of the rodent malaria parasites.
670

679 **REFERENCES:**
680 1. Who/Unicef Report: Malaria Mdg Target Achieved Amid Sharp Drop in Cases and Mortality,
681 but 3 Billion People Remain at Risk. *Neurosciences* (Riyadh). **21**, 87-88 (2016).
682
683 2. Naing, C., Whittaker, M. A., Nyunt Wai, V., Mak, J. W. Is Plasmodium vivax malaria a severe
684 malaria?: a systematic review and meta-analysis. *PLoS Neglected Tropical Diseases.* **8**, e3071
685 (2014).
686
687 3. Millar, S. B., Cox-Singh, J. Human infections with Plasmodium knowlesi--zoonotic malaria.
688 *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical*
689 *Microbiology and Infectious Diseases.* **21**, 640-648 (2015).
690
691 4. Spry, C., Kirk, K., Saliba, K. J. Coenzyme A biosynthesis: an antimicrobial drug target. *FEMS*
692 *Microbiology Reviews.* **32**, 56-106 (2008).
693
694 5. Aly, A. S., Vaughan, A. M., Kappe, S. H. Malaria parasite development in the mosquito and
695 infection of the mammalian host. *Annual Review of Microbiology.* **63**, 195-221 (2009).
696
697 6. Stephens, R., Culleton, R. L., Lamb, T. J. The contribution of Plasmodium chabaudi to our
698 understanding of malaria. *Trends in Parasitology.* **28**, 73-82 (2012).
699

700   7. Bagnaresi, P. *et al.* Unlike the synchronous Plasmodium falciparum and P. chabaudi infection,
701   the P. berghei and P. yoelii asynchronous infections are not affected by melatonin. *International*
702   *Journal of General Medicine.* **2**, 47-55 (2009).
703
704   8. Cromer, D., Evans, K. J., Schofield, L., Davenport, M. P. Preferential invasion of reticulocytes
705   during late-stage Plasmodium berghei infection accounts for reduced circulating reticulocyte
706   levels. *International Journal for Parasitology.* **36**, 1389-1397 (2006).
707
708   9. Jayawardena, A. N., Mogil, R., Murphy, D. B., Burger, D., Gershon, R. K. Enhanced expression
709   of H-2K and H-2D antigens on reticulocytes infected with Plasmodium yoelii. *Nature.* **302**, 623-
710   626 (1983).
711
712   10. Okada, H. *et al.* A transient resistance to blood-stage malaria in interferon-gamma-deficient
713   mice through impaired production of the host cells preferred by malaria parasites. *Frontiers in*
714   *Microbiology.* **6**, 600 (2015).
715
716   11. Walliker, D., Sanderson, A., Yoeli, M., Hargreaves, B. J. A genetic investigation of virulence in
717   a rodent malaria parasite. *Parasitology.* **72**, 183-194 (1976).
718
719   12. Deharo, E., Coquelin, F., Chabaud, A. G., Landau, I. The erythrocytic schizogony of two
720   synchronized strains of plasmodium berghei, NK65 and ANKA, in normocytes and reticulocytes.
721   *Parasitology Research.* **82**, 178-182 (1996).
722
723   13. Fahey, J. R., Spitalny, G. L. Virulent and nonvirulent forms of Plasmodium yoelii are not
724   restricted to growth within a single erythrocyte type. *Infection and Immunity.* **44**, 151-156 (1984).
725
726   14. Srivastava, A. *et al.* Host reticulocytes provide metabolic reservoirs that can be exploited by
727   malaria parasites. *PLoS Pathogens.* **11**, e1004882 (2015).
728
729   15. Hart, R. J. *et al.* Genetic Characterization of Plasmodium Putative Pantothenate Kinase Genes
730   Reveals Their Essential Role in Malaria Parasite Transmission to the Mosquito. *Scientific Reports.*
731   **6**, 33518 (2016).
732
733   16. Hart, R. J., Ghaffar, A., Abdalal, S., Perrin, B., Aly, A. S. Plasmodium AdoMetDC/ODC
734   bifunctional enzyme is essential for male sexual stage development and mosquito transmission.
735   *Biology Open.* **5**, 1022-1029 (2016).
736
737   17. Hart, R. J., Lawres, L., Fritzen, E., Ben Mamoun, C., Aly, A. S. Plasmodium yoelii vitamin B5
738   pantothenate transporter candidate is essential for parasite transmission to the mosquito.
739   *Scientific Reports.* **4**, 5665 (2014).
740
741   18. Ramakrishnan, C. *et al.* Laboratory maintenance of rodent malaria parasites. *Methods in*
742   *Molecular Biology.* **923**, 51-72 (2013).
743

744 19. Hart, R. J., Abraham, A., Aly, A. S. I. Genetic Characterization of Coenzyme A Biosynthesis
745 Reveals Essential Distinctive Functions during Malaria Parasite Development in Blood and
746 Mosquito. *Frontiers in Cellular and Infection Microbiology.* **7**, 260 (2017).
747
748 20. Vanderberg, J. P., Yoeli, M. Effects of temperature on sporogonic development of
749 Plasmodium berghei. *The Journal of Parasitology.* **52**, 559-564 (1966).
750
751 21. Vaughan, A. M., Aly, A. S., Kappe, S. H. Malaria parasite pre-erythrocytic stage infection:
752 gliding and hiding. *Cell Host & Microbe.* **4**, 209-218 (2008).
753
754 22. Briones, M. R., Tsuji, M., Nussenzweig, V. The large difference in infectivity for mice of
755 Plasmodium berghei and Plasmodium yoelii sporozoites cannot be correlated with their ability to
756 enter into hepatocytes. *Molecular and Biochemical Parasitology.* **77**, 7-17 (1996).
757
758 23. Hollingdale, M. R., Leland, P., Leef, J. L., Beaudoin, R. L. The influence of cell type and culture
759 medium on the *in vitro* cultivation of exoerythrocytic stages of Plasmodium berghei. *The Journal*
760 *of Parasitology.* **69**, 346-352 (1983).
761
762 24. House, B. L., Hollingdale, M. R., Sacci Jr., J. B., Richie, T. L. Functional immunoassays using an
763 *in vitro* malaria liver-stage infection model: where do we go from here? *Trends in Parasitology.*
764 **25**, 525-533 (2009).
765
766 25. Khan, Z. M., Vanderberg, J. P. Role of host cellular response in differential susceptibility of
767 nonimmunized BALB/c mice to Plasmodium berghei and Plasmodium yoelii sporozoites. *Infection*
768 *and Immunity.* **59**, 2529-2534 (1991).
769
770 26. Most, H., Nussenzweig, R. S., Vanderberg, J., Herman, R., Yoeli, M. Susceptibility of genetically
771 standardized (JAX) mouse strains to sporozoite- and blood-induced Plasmodium berghei
772 infections. *Military Medicine.* **131**, Suppl: 915-918 (1966).
773
774 27. Nussenzweig, R., Herman, R., Vanderberg, J., Yoeli, M., Most, H. Studies on sporozoite-
775 induced infections of rodent malaria. 3. The course of sporozoite-induced Plasmodium berghei
776 in different hosts. *The American Journal of Tropical Medicine and Hygiene.* **15**, 684-689 (1966).
777
778 28. Silvie, O., Franetich, J. F., Boucheix, C., Rubinstein, E., Mazier, D. Alternative invasion
779 pathways for Plasmodium berghei sporozoites. *International Journal for Parasitology.* **37**, 173-
780 182 (2007).
781
782 29. Tarun, A. S. *et al.* Protracted sterile protection with Plasmodium yoelii pre-erythrocytic
783 genetically attenuated parasite malaria vaccines is independent of significant liver-stage
784 persistence and is mediated by CD8+ T cells. *The Journal of Infectious Diseases.* **196**, 608-616
785 (2007).
786

787 30. Weiss, W. R., Good, M. F., Hollingdale, M. R., Miller, L. H., Berzofsky, J. A. Genetic control of
788 immunity to Plasmodium yoelii sporozoites. *The Journal of Immunology.* **143**, 4263-4266 (1989).
789

790 31. Philip, N., Orr, R., Waters, A. P. Transfection of rodent malaria parasites. *Methods in Molecular*
791 *Biology.* **923**, 99-125 (2013).
792

793 32. Janse, C. J., Ponzi, M., Sinden, R. E., Waters, A. P. Chromosomes and sexual development of
794 rodent malaria parasites. *Memorias do Instituto Oswaldo Cruz.* **89** Suppl 2, 43-46 (1994).
795

796 33. Sinha, A. *et al.* A cascade of DNA-binding proteins for sexual commitment and development
797 in Plasmodium. *Nature.* **507**, 253-257 (2014).
798

799 34. Malleret, B. *et al.* A rapid and robust tri-color flow cytometry assay for monitoring malaria
800 parasite development. *Scientific Reports.* **1**, 118 (2011).
801

802 35. Aly, A. S., Matuschewski, K. A malarial cysteine protease is necessary for Plasmodium
803 sporozoite egress from oocysts. *The Journal of Experimental Medicine.* **202**, 225-230 (2005).
804

805 36. Aly, A. S., Lindner, S. E., MacKellar, D. C., Peng, X., Kappe, S. H. SAP1 is a critical post-
806 transcriptional regulator of infectivity in malaria parasite sporozoite stages. *Molecular*
807 *Microbiology.* **79**, 929-939 (2011).
808

809 37. Aly, A. S. *et al.* Targeted deletion of SAP1 abolishes the expression of infectivity factors
810 necessary for successful malaria parasite liver infection. *Molecular Microbiology.* **69**, 152-163
811 (2008).
812

813 38. Ozaki, L. S., Gwadz, R. W., Godson, G. N. Simple centrifugation method for rapid separation
814 of sporozoites from mosquitoes. *The Journal of Parasitology.* **70**, 831-833 (1984).
815

816 39. de Koning-Ward, T. F., Gilson, P. R., Crabb, B. S. Advances in molecular genetic systems in
817 malaria. *Nature Reviews. Microbiology.* **13**, 373-387 (2015).
818

819 40. Janse, C. J., Ramesar, J., Waters, A. P. High-efficiency transfection and drug selection of
820 genetically transformed blood stages of the rodent malaria parasite Plasmodium berghei. *Nature*
821 *Protocols.* **1**, 346-356 (2006).
822

823 41. Lin, J. W. *et al.* A novel 'gene insertion/marker out' (GIMO) method for transgene expression
824 and gene complementation in rodent malaria parasites. *PLoS One.* **6**, e29289 (2011).
825

826 42. Manzoni, G. *et al.* A rapid and robust selection procedure for generating drug-selectable
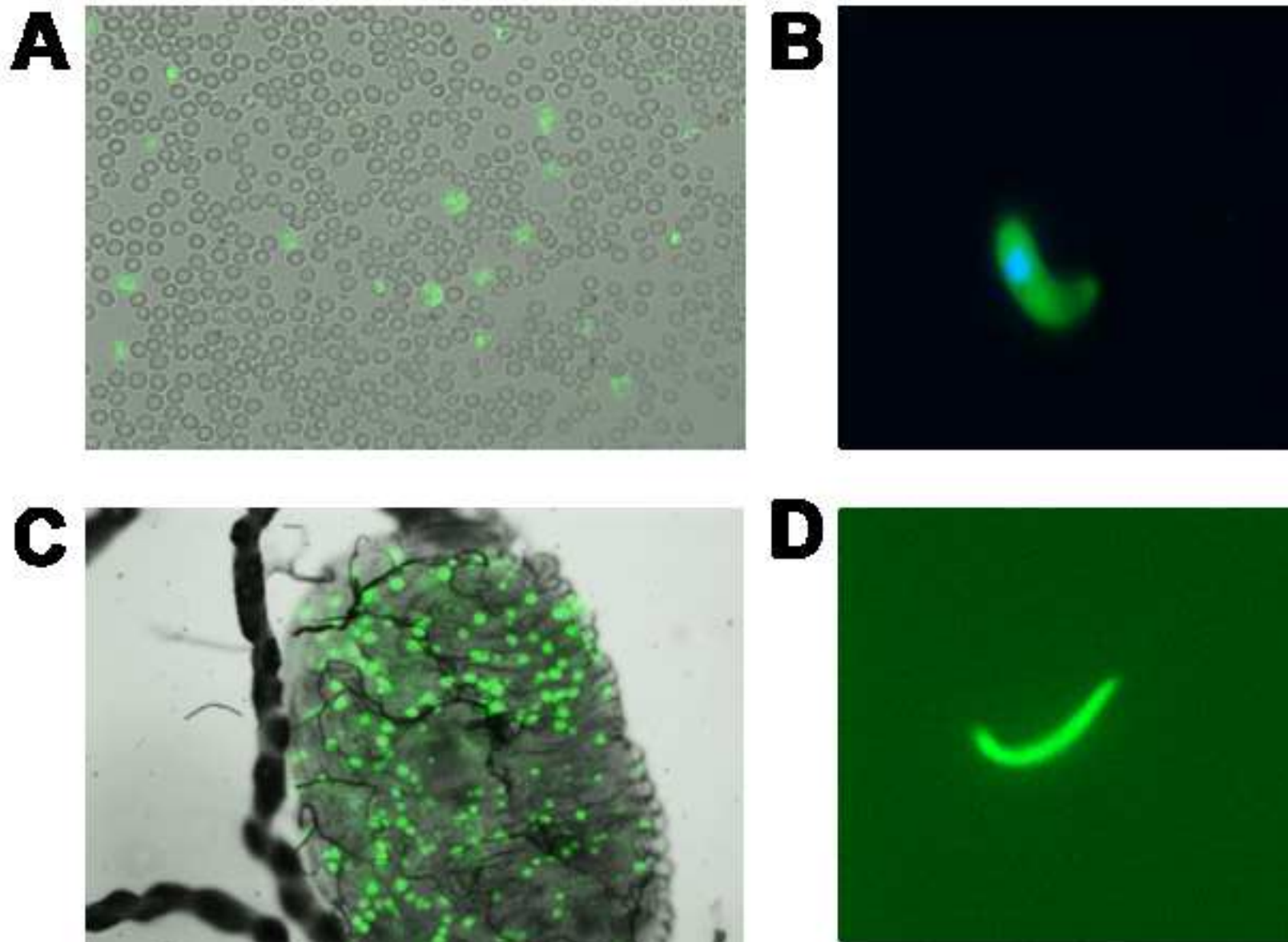**827** marker-free recombinant malaria parasites. *Scientific Reports.* **4**, 4760 (2014).
828

Figure 1

Figure 1

Figure 2

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Figure 2

Figure 3

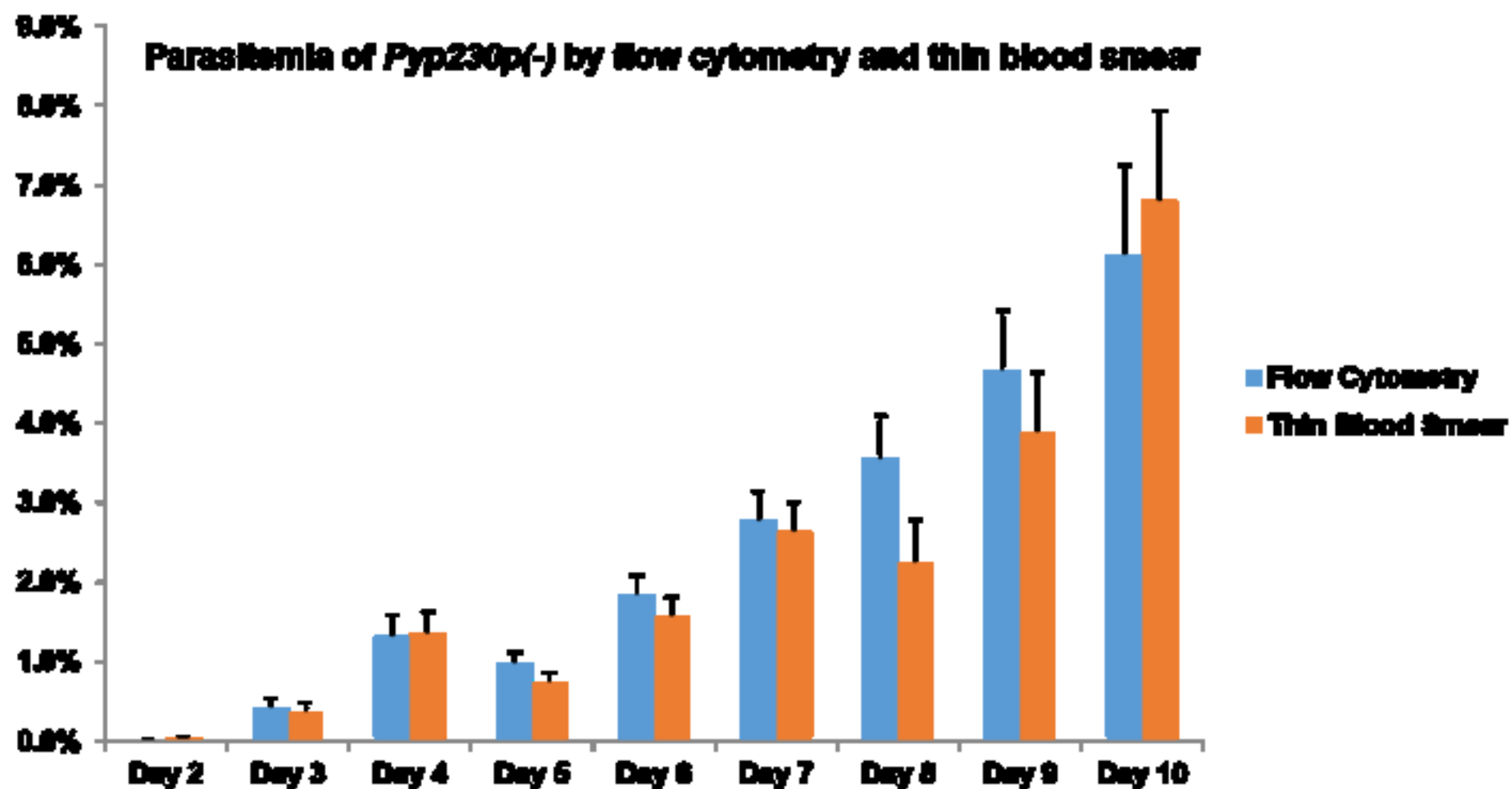Click here to access/download;Figure;Figure3new.tif ⬇

Figure 3

Parasitemia of Intravenous vs. Intraperitoneal infection with *Pyp230p(-)*

Figure 4

Figure 4

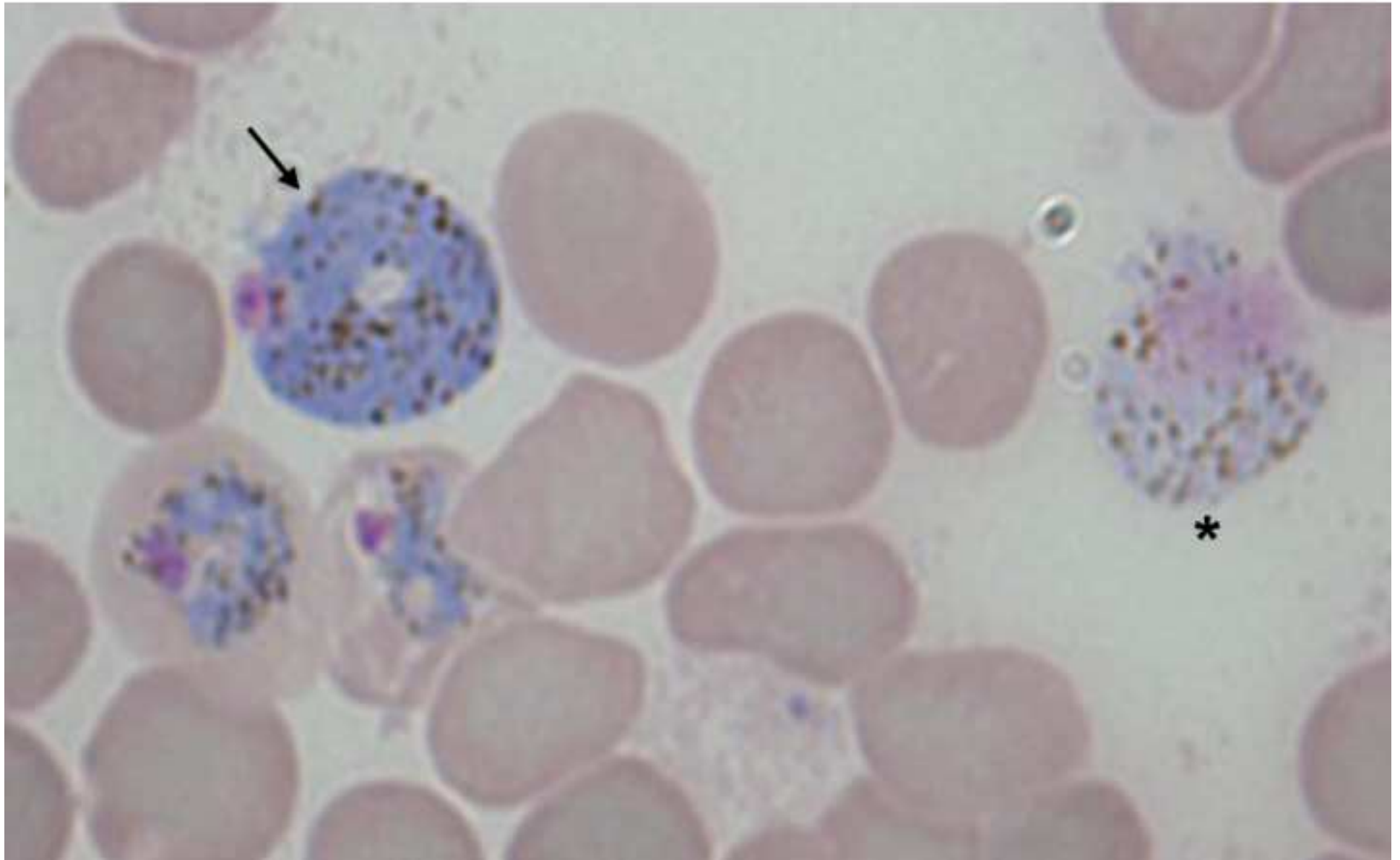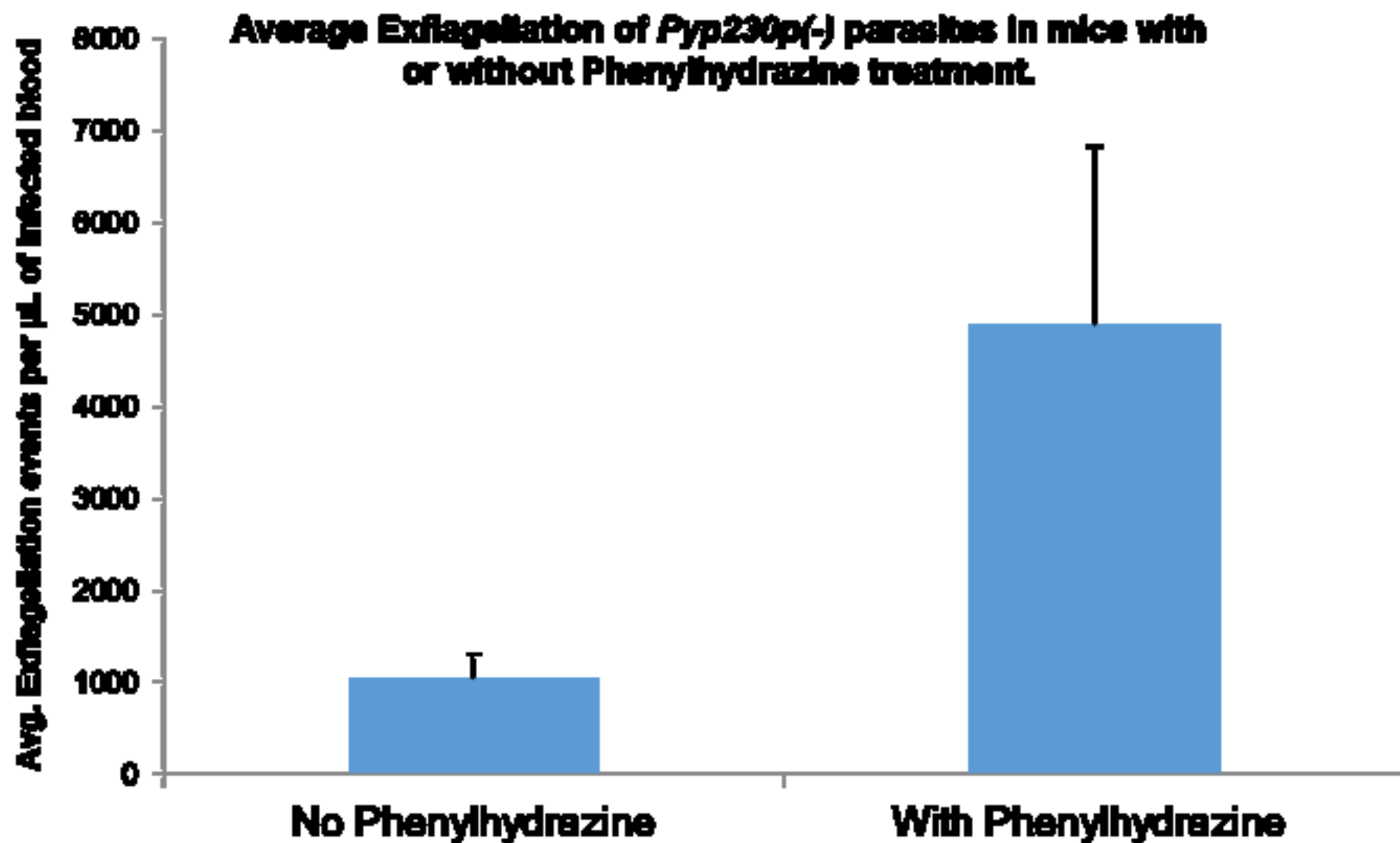Figure 5

Figure 5

Average Exflagellation of *Pyp230p(-)* parasites in mice with or without Phenylhydrazine treatment.
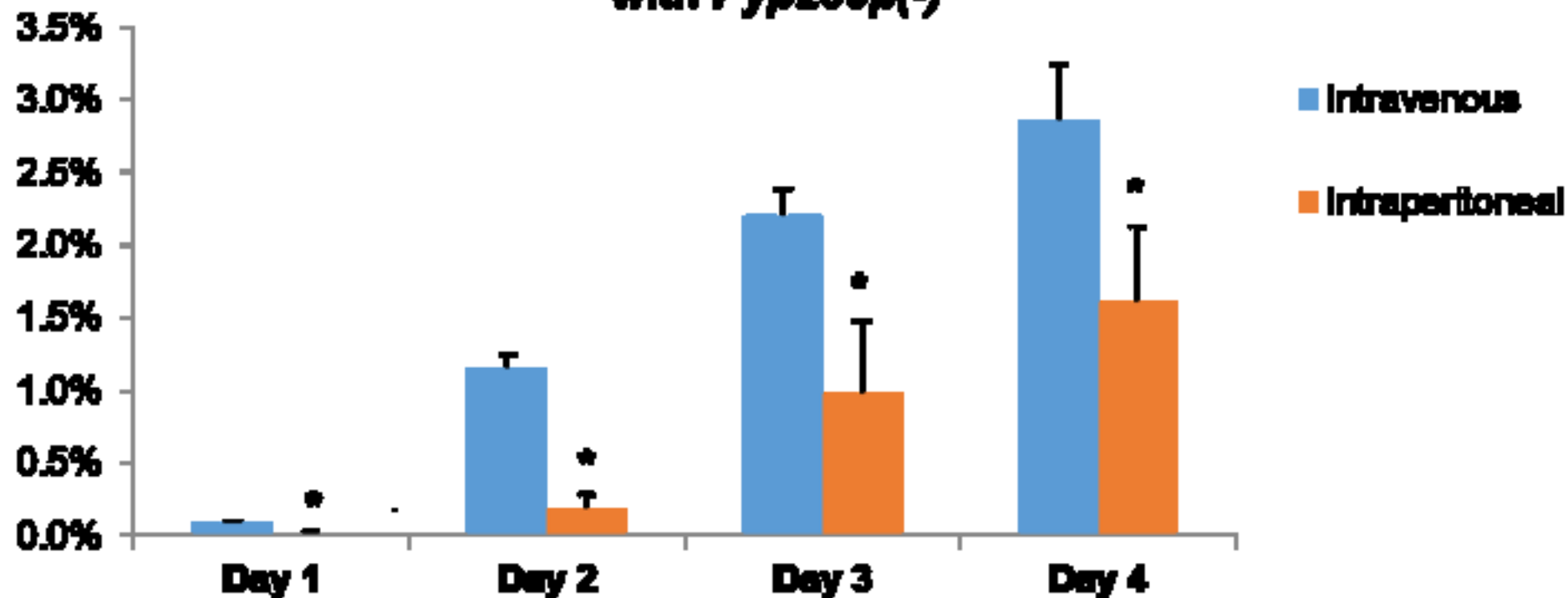
Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Heparin	Sigma	375095-100KU	
Xanthurenic acid	Sigma	D120804-5G	
Hypoxanthine	Sigma	H9377-25G	
Alsever's solution	Sigma	A3551-500ML	
Sodium Bicarbonate	Sigma	S5761-500G	
Phenylhydrazine	Sigma	P26252-5G	
Glycerol	Sigma	G5516-500ML	
Giemsa	Sigma	GS1L-1L	
26G x 3/8 Precision Glide Needle,	Becton Dickinson	305110	
1 ml TB Syringe, 26G x 3/8	Becton Dickinson	309625	
1 cc Insulin Syringe, U-100 27G	Becton Dickinson	329412	
Isoflurane, USB	Piramal	2667- 46- 7	
PBS, pH 7.4	Gibco	10010049	
RPMI	Gibco	22400105	
DMEM	Gibco	11995065	
Pencillin/ Streptomycin	Gibco	10378016	
Fetal Bovine Serum	Gibco	10082147	
Fiber Glass Wool	Corning	3950	

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August 15th, 2018

Dear JOVE Editor,

Please, find enclosed our revised manuscript entitled "**Phenotypic Analysis of Rodent Malaria Parasite Asexual and Sexual Blood Stages and Mosquito Stages.**" for publication consideration in JOVE. **In the revised manuscript, we fully addressed all concerns of the scientific review editor, which were provided to us as comments in the manuscript file, as replies to the same comments in the same manuscript file.** We tracked all changes in the manuscript and we highlighted the sections that we wish to be filmed.

We hope to hear a positive response from you soon.

Sincerely,
Ahmed Aly (on behalf of all the authors)

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