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1 **Splenic retention of *Plasmodium falciparum* gametocytes**

2 **to block the transmission of malaria**

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17 **Running title :** *Gametocyte stiffening to block malaria transmission*

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19

20 **ABSTRACT:**

21

22 **Background:** *Plasmodium falciparum* is transmitted from humans to *Anopheles* mosquito  
23 vectors via the sexual erythrocytic forms termed gametocytes. Erythrocyte filtration through  
24 microsphere layers (microsphiltration) had shown that circulating gametocytes were  
25 deformable. Compounds reducing gametocyte deformability would induce their splenic  
26 clearance, thus remove them from the blood circulation and block malaria transmission.

27

28 **Methods:** The hand-made, single-sample prototype for microsphiltration was miniaturized to  
29 a 96-well microtitre plate format, and gametocyte retention in the microsphere filters  
30 quantified by high-content imaging. The stiffening activity of 40 pharmacological compounds  
31 was assessed in microtitre plates, using a small molecule (calyculin) as a positive control. The  
32 stiffening activity of calyculin was assessed in spleen-mimetic microfluidic chips and in  
33 macrophage-depleted mice.

34

35 **Results:** Marked mechanical retention (80-90%) of mature gametocytes was obtained in  
36 microplates following exposure to calyculin at concentrations with no effect on parasite  
37 viability. Of the 40 compounds tested, including 20 anti-malarials, only 5 endoperoxides  
38 significantly increased gametocyte retention (1.5–2.5 fold; 24 hour-exposure at 1  $\mu$ M).  
39 Mature gametocytes exposed to calyculin accumulated in microfluidic chips, and were cleared  
40 from the circulation of macrophage-depleted mice as rapidly as heat-stiffened erythrocytes,  
41 thus confirming results obtained using the microsphiltration assay.

42

43 **Conclusions:** An automated miniaturized approach to select compounds for their gametocyte-  
44 stiffening effect has been established. Stiffening induces gametocyte clearance both *in vitro*  
45 and *in vivo*. Based on physiologically validated tools, this screening cascade can identify  
46 novel compounds and uncover new targets to block malaria transmission. Innovative  
47 applications in hematology are also envisioned.

48

49 Key words: malaria; *Plasmodium falciparum*, transmission, deformability, spleen, clearance,  
50 screening, microspheres, gametocytes, high content imaging.

51

52 **INTRODUCTION:**

53 Every 2-3 hours, human red blood cells (RBC) enter the open and slow microcirculation of  
54 the spleen where they cross 2  $\mu\text{m}$ -wide inter-endothelial slits before returning to the general  
55 circulation [1]. In several inherited or acquired diseases, RBC are unable to deform  
56 sufficiently to overcome this mechanical challenge [2]. Their splenic retention subsequently  
57 induces anemia and splenomegaly [3]. *Plasmodium falciparum* (*Pf*)-malaria is the most  
58 frequently acquired disease where RBC deformability is affected [4]. Intra-erythrocytic  
59 asexual parasitic stages develop during a 2-day replication cycle, where rings evolve to multi-  
60 nucleated schizonts that rupture the host RBC, thus releasing daughter merozoites which re-  
61 invade new RBC [4]. Asexual maturation results in extensive RBC remodeling [5] through  
62 parasite growth and exported proteins which restructure the RBC and trigger stiffening [6].  
63 Rings are modestly deformable, a proportion of them circulate [7]. Mature-asexual stages are  
64 rigid and sequester in micro-vessels where they are protected from splenic mechanical  
65 clearance [8]. *Pf* transmission from humans to mosquitoes relies on the conversion of a small  
66 fraction of asexual parasites to sexual stages, called gametocytes [9]. *P. falciparum*  
67 gametocytes develop throughout a 2-week period during which immature stages are  
68 sequestered, predominantly in the extravascular compartment of the bone marrow [10, 11].  
69 Remodeling of the erythrocyte and parasite membrane also occurs during sexual development  
70 [12, 13]. Stiff, immature gametocytes become deformable mature gametocytes that are  
71 released into the peripheral circulation [14, 15]. Circulating mature gametocytes must then  
72 repeatedly cross splenic inter-endothelial slits. Their ability to traverse these slits is essential  
73 for their persistence in circulation and availability to mosquitoes [1].

74 One strategy towards *Pf*-malaria elimination focuses on the discovery of compounds blocking  
75 gametocyte transmission from humans to mosquitoes. Several screening assays have been

76 reported [1, 16-24] which identified small molecules that kill gametocytes or affect their  
77 development in the mosquito. To expand the repertoire of transmission-blocking compounds  
78 and uncover new modes of action, we propose a unique screening approach to identify  
79 compounds that will stiffen mature gametocytes, thus triggering their splenic mechanical  
80 retention [25]. Once cleared from the circulation, mature gametocytes will be removed from  
81 the transmission cycle.

82 Most existing methods to study the deformability of *Pf*-infected RBC [7, 26, 27] are not yet  
83 compatible with the stringent requirements for high throughput screening (HTS). A single-  
84 sample microsphere filtration (microsphiltration) device to measure the ability of RBC to  
85 squeeze between calibrated microspheres, thus mimicking the spleen, has been recently  
86 developed [28]. The physiological relevance of microsphiltration was demonstrated by  
87 obtaining similar retention rates for abnormally deformable RBC in the device and in human  
88 spleens perfused *ex-vivo* [28]. We report here on the development and validation of a  
89 microplate version of the microsphiltration device. Microplate filtration is well adapted to  
90 screen for compounds that will stiffen mature gametocytes. Pharmacological stiffening  
91 observed in the microsphiltration assay translated into gametocyte entrapment in microfluidic  
92 chips and in their clearance from the circulation of macrophage-depleted mice. Based on these  
93 physiologically validated tools, this approach can identify novel compounds and uncover new  
94 targets to block malaria transmission.

95

## 96 **MATERIALS AND METHODS:**

97 *Preparation of microsphiltering microplates*

98 Calibrated microsphere mixtures (AMTech® 96.5% tin, 3% silver, 0.5% copper), 25-45µm  
99 and 5-15µm were resuspended at 2 and 3g/mL, respectively, in phosphate buffered saline  
100 (PBS, Sigma) supplemented with 0.5% Albumax II (Life Technologies), then allowed to  
101 settle. Fifty µL of 25-45µm microsphere solution was transferred to 96-deep well filter  
102 bottom plate (Harvard Apparatus) using a Biomek 3000 liquid handler (Beckman Coulter),  
103 then 60µL of 5-15µm microspheres were added, resulting in an ~1.15mm-thick layer.

#### 104 *Parasite culture*

105 The *Plasmodium falciparum*, NF54-derived Pfs16-luciferase-GFP transgenic clone 3 [29],  
106 was cultured as described [16, 30] using RPMI 1640 with L-Glutamine and 25mM 4-(2-  
107 hydroxyethyl)-1-piperazine-ethane-sulfonic acid (HEPES) supplemented with 2.5mg/ml  
108 Albumax II (Life Technologies), 5% AB+ Male Human Serum, 50µg/mL of Hypoxanthine  
109 and 2µg/ml Blasticidin (Sigma). Gametocyte growth media without blasticidin was  
110 supplemented with 0.5M N-acetyl-glucosamine (NAG) and 11mM Glucose (Sigma). Highly  
111 synchronous gametocyte cultures were induced from asexual cultures [16] within a 12 hour  
112 window at the outset of gametocytogenesis and uniformly differentiated Stage V gametocytes  
113 were harvested at day 12 or 13 post-induction for deformability experiments. For selected  
114 mouse experiments, mixtures of immature and mature gametocytes were used.

#### 115 *Light microscopy*

116 Thin blood films were fixed in 100% methanol, stained with Giemsa (Sigma) and counted  
117 using 100X oil immersion objective and bright-field illumination. The percentage sexual or  
118 asexual stages was counted against  $10^4$  RBC.

#### 119 *PKH26 and PKH67-labeling of normal and heated RBC*

120 RBC were washed with PBS and heated at 50°C for 20 minutes. Heated and control RBC  
121 were labeled using PKH26 or PKH67 Fluorescent Cell Linker Kit for General Cell Membrane  
122 Labeling (Sigma Aldrich) as described [28]. PKH-labeled RBC were resuspended in assay  
123 buffer (for asexual) or PBS-Albumax II 1% and mixed 1:20 with unlabeled RBC.

#### 124 *Flow cytometry*

125 PKH-labeled RBC were quantified with a BD Accuri C6 flow cytometer using 488nm (PKH-  
126 67) and 640nm (PKH-26) laser excitation. Cells were resuspended in PBS at  $10^6$ /mL and  
127 parasitemia determined from acquisition of  $>30 \times 10^3$  events. Data were collected and  
128 processed using the BD Accuri C6 software.

#### 129 *Microfiltration*

130 Following loading with stage V mature gametocyte (200 $\mu$ L per well at 2% hematocrit, 5%  
131 gametocytemia) or control samples, microtitre plates were vacuum aspirated using a manifold  
132 system (Beckman Coulter) coupled to an electric High Output vacuum pump (Millipore) via a  
133 10L trap. Sample aspiration (peak pressure of 7 In.Hg) was followed by a rinsing step with  
134 1.6mL of gametocyte culture medium without NAG (vacuum aspiration, peak pressure of 10  
135 In.Hg). The downstream sample of 1.8mL was retrieved and RBC populations quantified.

#### 136 *Compound Testing*

137 Forty-compounds consisting of 20 FDA-approved drugs and 20 antimalarial agents were  
138 selected for assay validation. Two hundred  $\mu$ L of mature gametocyte culture at day 11 post-  
139 induction, 2.5% hematocrit and ~3-5% parasitemia were dispensed into polypropylene plates  
140 containing compounds at 5 $\mu$ M or 0.5 $\mu$ M in 50 $\mu$ L of gametocyte growth media and final 0.4%  
141 dimethyl sulfoxide concentration (DMSO), then incubated with gas permeable sealed

142 membranes (Corning) for 24h in a 37°C incubator. As negative and positive control,  
143 gametocytes were exposed to 50nM Calyculin, a phosphatase inhibitor without effect on the  
144 deformability of uninfected RBC [31] or 0.4% DMSO, respectively.

145 *Opera high content imaging*

146 Following microfiltration, 4.5µL (approximately  $10^5$  RBC) of each filtered sample and  
147 their corresponding unfiltered control diluted to a final hematocrit of 0.2% were transferred  
148 using an Agilent Bravo liquid handler to 384-well CellCarrier imaging plates (Perkin Elmer)  
149 containing 45.5µL of Cell Mask Plasma Membrane Orange® stain (Life Technology) diluted  
150 1:13,333 in PBS.

151 Quantification of gametocytemia was determined using high content confocal imaging  
152 (Opera™, Perkin Elmer) through acquisition of 17 images per well using a 20X water  
153 immersion objective (NA 0.7) resulting in at least  $10^4$  erythrocytes per well. GFP-positive  
154 gametocytes were detected using 488nm excitation with 520/35nm emission and CellMask  
155 Orange labelled erythrocytes were detected with 560nm excitation and 600/40 emission.

156 *Data analysis*

157 Images were processed using the Opera™ Harmony® software (PerkinElmer) using image  
158 segmentation algorithms. Gametocytes were classified and counted based on elongation  
159 index, size and GFP intensity. RBC were detected using CellMask Orange staining intensity,  
160 circularity and size. Gametocytemia (up- and downstream) was defined as the total number of  
161 gametocytes detected divided by that of erythrocytes, and the corresponding retention rate  
162 was calculated as described [28]. Significance probability for compound-induced gametocyte



163 stiffening was calculated using non-parametric, U-statistic Mann Whitney test with two tailed  
164 P value and confidence interval of 99%.

#### 165 *Microfluidics*

166 The microfluidic biochips were cast in polydimethylsiloxane (PDMS, Sylgard, France), a  
167 silicone elastomer produced as described [32]. Each biochip comprised 8 parallel filtering  
168 units connected to infusion tubing. Biochips were coated with PBS 1% albumin before all  
169 experiments. Cultures containing 5% mature gametocytes at 2% hematocrit were exposed to  
170 Calyculin (50 nM) or control DMSO 0.4% for 2 hours prior labeling with Hoechst or Sybr  
171 Green (Life Technologies), mixing in a 1:1 ratio and centrifugation. RBC samples were  
172 infused using a push-syringe as 100% RBC concentrate of 20 $\mu$ L eluted with PBS-Albumax II  
173 1% under a 200 (+/- 50)  $\mu$ L/hour flow rate and 150 (+/- 50) mbar pressure. Enrichment of  
174 mature gametocytes in the slits of each filtering units was quantified from fluorescent  
175 microscope images (Leica DMI3000 microscope, using Leica DFC310FX camera controlled  
176 LAS Superposition Image software, Leica Micro-système, Nanterre, France) acquired 15  
177 minutes post-infusion. Counting was performed by 3 independent unbiased evaluators.

#### 178 *Splenic mechanical retention of gametocytes in mice*

179 All in vivo experiments were performed in accordance with the protocols of the Pasteur  
180 Institute (Paris) and the Guidelines for the Care and Use of Laboratory Animals [33].  
181 Experiments were performed with C57BL6 adult mice (8-12 weeks) injected or not with  
182 100 $\mu$ L clodronate liposome (ClodronateLiposomes.com®, Netherlands) for macrophage  
183 depletion 24 and 48 hours prior to the transfusion of human cells. Mice were injected retro-  
184 orbitally with 100 $\mu$ L human RBC suspensions at 20% hematocrit (in culture media)  
185 containing 40% of heat-stiffened RBC (HRBC) or immature (3-10 days post-induction) or

186 mature (12-14 days post-induction) *P. falciparum* gametocytes (NF54 or Pf16). For  
187 assessment of calyculin stiffening activity, mature gametocyte cultures were exposed for 2  
188 hours to either calyculin (100nM) or control DMSO (0.4%) before injection in clodronated  
189 mice. For control experiments, HRBC (50°C, 20 minutes) or control unheated RBC (nRBC)  
190 were differentially labeled with PKH-26 and PKH-67, respectively, prior to injection. Blood  
191 samples were then collected from the tail vein 1, 90 and 180 minutes post-injection.  
192 Circulating concentrations of heated and unheated human RBC were determined by flow  
193 cytometry. The concentration of mature and immature gametocytes was determined by  
194 microscopic examination of Giemsa-stained blood films.

195

196 **RESULTS:**

197 **96-well microfiltration mimics splenic retention of *Pf* infected and abnormal RBC.**

198 To improve throughput, the tip-based microfiltration method [28] was adapted to a 96-well  
199 microplate format. Deep-well plates designed for vacuum-based filtration were prepared with  
200 two layers of microspheres, designed to replicate the filtering properties of the tip-based  
201 approach. Optimal dispensing of microspheres into the plates was standardized using an  
202 automated liquid dispenser (Fig 1-A, b). A two-step vacuum-based aspiration protocol was  
203 validated (Figure 1-B). The microsphere matrix remained wet between filtration steps, and the  
204 morphology of filtered RBC was preserved (Fig 1D).

205 Mimicry of splenic mechanical sensing was confirmed by quantifying the retention rates of  
206 uninfected RBC and *Pf* asexual blood stages. As expected, weak retention (<10%) was  
207 observed for normal RBC (Figure 1-C). Heat-stiffened RBC [35] were retained by more than  
208 90%. As previously observed with the tip-based prototype, ring stage *Pf*-infected RBC  
209 showed moderate retention at  $35.8 \pm 10.7\%$ , while mature asexual stages were retained at  $81.5$   
210  $\pm 5.1\%$  (Fig 1-C). The relatively wide distribution of retention rates for rings was related to  
211 their increasing loss in surface area to volume ratio as the parasite matures [35,36] and the  
212 distribution of ages across different experiments. These retention rates represent a dynamic  
213 range of filtering capability consistent with previously published results [28] using the single-  
214 sample prototype or *ex vivo* human spleen perfusion [37]. These data illustrate the successful  
215 development of microplate-based microfiltration for rapid and robust determination of  
216 spleen-like retention of RBC samples.

217 **Increased throughput and automated quantification of gametocytes**

218 Retention rates of RBC harbouring asexual *Pf* stages in the microfiltration assay were  
219 determined using Sybr green and flow cytometry analysis. Retention rates calculated either  
220 from Giemsa-stained blood films or flow cytometry showed a strong linear correlation ( $r^2 \geq$   
221 0.9; Fig 2-A). Retention rates of gametocytes was determined using high content imaging  
222 based on RBC labelling with a fluorescent membrane stain and gametocyte GFP expression  
223 [29]. A strong linear correlation ( $r^2 = 0.96$ ) was obtained between manual counting using a  
224 hemocytometer and imaging-based quantification, across the full range of mature gametocyte  
225 concentrations relevant for this assay (Figure 2B).

226

#### 227 **Proof of concept for pharmacological screening using microfiltering microplates**

228 Using the microfiltration assay, untreated or DMSO-exposed, mature gametocytes  
229 exhibited less than 50% retention (Figure 3-A). Exposure to 50nM calyculin for 2 and 24  
230 hours resulted in a significant increase in retention to  $89.85 \pm 5.17\%$  and  $79.26 \pm 3.21\%$  ( $p <$   
231 0.0001), respectively. Evaluation of calyculin gametocytocidal activity using a mitotracker-  
232 based viability assay [17] showed a  $<25\%$  inhibition at 100nM (Fig 3-A). The difference in  
233 retention rates between DMSO and calyculin-exposed gametocytes provided a screening  
234 window stable across 3 independent biological replicates, with an average  $Z'$  score of 0.67.

235 Forty compounds, including 20 anti-malarial agents and 20 FDA-approved drugs were tested  
236 using the assay. None of the 20 FDA-approved drugs significantly increased gametocyte  
237 retention above negative control values. Of the 20 anti-malarial agents tested with a 24-hour  
238 exposure, only 5 endoperoxide derivatives, namely artemether, dihydro-artemisimin (DHA),  
239 artesunate (ART), artemisinin and artemisone were found active at  $1\mu\text{M}$  ( $p < 0.0001$ ) (Fig 3-  
240 B, C, D). Assessment of gametocytocidal activity for these compounds revealed killing  
241 activity at concentrations lower than those inducing mechanical retention (Supplementary Fig

242 S1). The microtitre plate-based microsphiltration platform thus determined the gametocyte-  
243 stiffening effect of multiple compounds in parallel.

#### 244 **Physiological relevance of the screening process**

245 To confirm that a compound identified as active in the microsphiltration assay induces the  
246 mechanical blockade of compound-exposed gametocytes in the spleen, we analyzed the fate  
247 of gametocytes treated with calyculin *in vitro* and *in vivo*.

248 *Mechanical blockade in a spleen-mimetic microfluidic chip:* Mature gametocytes from 2  
249 different laboratory strains and a sample directly collected from the blood of a *P. falciparum*-  
250 infected-patient were exposed to calyculin at 50 nM for 2 hours, then infused through  
251 microfluidic chips. This method has been shown to recapitulate the mechanical constraints  
252 imposed on RBC as they cross inter-endothelial slits in the human spleen and validated with  
253 an array of poorly deformable RBC [32]. Exposure to calyculin induced a marked enrichment  
254 to  $72.40 \pm 13.64\%$  in mature gametocytes in the narrowest slits of the chips versus  $27.60 \pm$   
255  $13.64\%$  for the DMSO-exposed gametocytes (Fig 4A) ( $p < 0.0001$ ). Thus, the altered  
256 mechanical behavior of pharmacologically-stiffened mature gametocytes observed with the  
257 microsphiltration assay was confirmed using microfluidic chips as an independent measure of  
258 spleen-like retention. This provides a physiologically relevant validation of active compounds  
259 identified by microsphiltration.

260 *Clearance of pharmacologically-stiffened gametocytes from the blood of macrophage-*  
261 *depleted mice:* Gametocytes exposed to either calyculin or 0.4% DMSO were transfused into  
262 C56Bl6 mice previously treated with clodronate liposomes to deplete endogenous  
263 macrophages and allow normal human RBC to circulate for several hours (Fig 4B, C and D).  
264 As expected, when untreated mice were transfused with human RBC a complete clearance

265 was observed in less than 90 minutes. By contrast,  $73.19 \pm 26.87\%$  and  $58.53 \pm 12.97\%$  of  
266 human RBC ( $p = 0.0006$ ) were still in circulation at 90 and 180 minutes, respectively, after  
267 transfusion into macrophage-depleted mice. In these macrophage-depleted mice, only  $35.63 \pm$   
268  $24.42\%$  and  $22.98 \pm 6.94\%$  of stiff, heat treated RBC remained in circulation after 90 and 180  
269 minutes, corresponding to  $>75\%$  clearance, arguably by a macrophage-independent,  
270 mechanical process (Fig 4B). When gametocyte circulation kinetics were examined, we found  
271 that  $81.21\%$  of immature compared to  $15.35\%$  of mature gametocytes were cleared after 90  
272 minutes ( $p = 0.0005$ ). This result is consistent with previous studies which demonstrated a  
273 marked difference in the stiffness of immature and mature stages using biorheological  
274 methods [14, 15] (Fig 4C). Finally, mature gametocytes exposed to calyculin for 2 hours had  
275 a clearance rate of  $77.52\%$ , whereas exposure to  $0.4\%$  DMSO resulted in only  $34.83\%$   
276 retention after 180 minutes ( $p = 0.0286$ ). Interestingly, these clearance rate kinetics were  
277 almost identical with those of nRBC ( $26.97\%$ ) and HRBC ( $77.01\%$ ). Hence, a compound  
278 active in the microsphere filtration assay induces the splenic mechanical retention of gametocytes  
279 in vivo (Fig 4D).

280

281 **Discussion:**

282 Using a miniaturized microfiltration assay designed to mimic the mechanical sensing of  
283 RBC by the spleen, we have demonstrated that a small molecule can significantly impair the  
284 ability of mature *P. falciparum* gametocytes to cross spleen-like slits, independently from  
285 gametocyte killing. This finding was confirmed *in vitro* using biomimetic microfluidic chips,  
286 and *in vivo* using a macrophage-depleted mouse model. Collectively, these results establish an  
287 original screening and post-screening cascade to select compounds that induce the mechanical  
288 retention of mature gametocytes in the human spleen, thus eliminating them from the malaria  
289 transmission cycle. Once retained in the spleen, the fate of a RBC is macrophage-mediated  
290 phagocytosis and destruction. Mechanical retention of RBC is effective in malaria patients  
291 [1] [34]. Therefore, compounds which induce retention and clearance of mature gametocytes  
292 in the spleen represent solid candidates as transmission-blocking agents.

293 To find transmission-blocking agents through an original, biomechanical approach we  
294 optimized the manual microfiltration prototype to a miniaturized automated format. This  
295 effectively streamlined the processing of 96 samples in a simultaneous fashion with the  
296 potential to screen up to 5,000-10,000 compounds in single point, over a 1 to 2-month period.  
297 We have also developed specific tools to validate this approach. Lavazec and colleagues  
298 recently identified compounds interfering with a signalling pathway that regulates  
299 gametocyte-infected RBC deformability. The identification of calyculin [31] has provided the  
300 much needed tool for validation of the screening approach reported here. We have  
301 demonstrated that calyculin stiffens gametocytes at concentrations lower than those affecting  
302 gametocyte viability (Fig 3). This stiffening activity was further confirmed using biomimetic  
303 microchips with narrow 2 micron-wide slits [32] in which gametocytes exposed to calyculin  
304 accumulated after a few minutes of infusion (Fig 4A). To validate the effect of hits *in vivo*,

305 we developed a new transfusion approach in mice. The mouse and human spleens retain  
306 autologous spherocytic RBC at a similar pace [38, 34] and the minimal surface area loss  
307 triggering biomechanical retention is also similar in both species, namely >18% in human  
308 spleens [35] and >20% in mice [38]. Macrophage depletion was used to allow human RBC to  
309 remain in the peripheral circulation of C57BL6 mice. As expected, those human RBC were  
310 rapidly cleared from the circulation of macrophage-intact controls (Fig 4B), while the  
311 majority of them were still in the peripheral blood of macrophage-depleted mice 3 hours after  
312 xenotransfusion. In this model, a minority of mature gametocytes and a majority of immature  
313 gametocytes were cleared, consistent with previous observations *in vitro* [14]. Similarly, only  
314 a minority of mature gametocytes exposed to DMSO and a majority of those exposed to  
315 calyculin were cleared. This strongly suggests that calyculin induces the clearance of mature  
316 gametocytes *in vivo* by inducing their mechanical retention in the spleen [33, 40].

317 In the first set of compounds tested, most were inactive and induced retention rates very  
318 similar to those observed with the solvent control (DMSO) (Fig 3). Five endoperoxides were  
319 identified as actives, however, as opposed to calyculin, induced retention only at  
320 concentrations higher than those affecting viability (Fig 3 & Fig S1). Mechanical retention  
321 was thus more likely a consequence of gametocyte killing rather than a new mode of action of  
322 endoperoxides on gametocyte carriage. Interestingly, artemether affected the viability of  
323 mature gametocytes at lower concentrations than dihydroartemisinin. This is consistent with  
324 the greater effect artemether-lumefantrine has on gametocyte carriage and transmission to  
325 *Anopheles* compared to DHA-piperaquine [41].

326 The ideal compound selected by our screening/post-screening cascade would induce a potent,  
327 irreversible increase in the retention of mature gametocytes and have a long half-life.  
328 Duration of gametocyte carriage is reduced from 7 to 2 weeks in malaria patients treated with



329 ACTs [42, 43]. A mean duration of activity of two weeks would thus cover the gametocyte  
330 circulation period in ACT-treated patients. What is the minimal activity that would translate  
331 into public health benefit? Modeling indicates that the prediction is complex, due to a possibly  
332 non-linear relationship between gametocytemia and transmission to Anopheles [44] and a  
333 wide diversity of epidemiological situations. While moderately active compounds (eg, 80%  
334 retention) would impact transmission from subjects carrying >250-300 gametocytes/ $\mu$ l, only  
335 solidly active compounds (> 98% retention) would have an impact in subjects with <20  
336 gametocytes/ $\mu$ l. Whatever the anti-gametocyte approach used, reducing transmission from  
337 subjects with intermediate gametocytemia (20-200 gametocytes/ $\mu$ l) will require very potent  
338 activity. If modeling accurately reflects reality, combining a gametocyte-killing agent from  
339 ongoing screening activities [16-24] with a gametocyte-stiffening agent from our  
340 biomechanical approach may increase the likelihood for transmission-blocking success.

341 Following assay optimization, microplate-based microsphere-based microsphere filtration proved robust and flexible.  
342 The results were reproducible across multiple microplate formats, utilizing various automated  
343 dispensers, different microsphere sources and microsphere filtration media. Optimization by  
344 analyzing male/female and reversible/irreversible effects is envisioned. We have developed a  
345 storage process that preserves the integrity of the microsphere layer and assay performance  
346 for 3 months thereby enabling transport and deferred use at distant laboratories (not shown).  
347 Potential applications include HTS to discover compounds modulating the biomechanical  
348 properties of RBC in conditions such as diabetes [45], sepsis and inflammatory diseases [46],  
349 inherited RBC disorders [47], or transfusion [48]. The assay may also provide a quality  
350 control test for RBC concentrates, as poorly deformable subpopulations of RBC tend to  
351 accumulate upon storage [28, 49].

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- 531

532 **FIGURE LEGENDS**

533 **Figure 1: From the hand-made, single-sample prototype to a high throughput**  
534 **microfiltration assay using microplates.** (A) In the prototype, 5 – 25  $\mu\text{m}$  microspheres  
535 were directly deposited by hand above the anti-aerosol filter of a tip. In the microplate assay,  
536 25 to 45 $\mu\text{m}$ -diameter microspheres [2] were poured using automated dispenser into 96-well  
537 filter mesh plates to support a 1.15 mm-thick layer of 5-15 $\mu\text{m}$  microspheres [1] forming the  
538 matrix mimicking the geometry of splenic slits [28]. (B) The driving force for  
539 microfiltration in the Tip format was applied using syringes connected to hermetic tubing.  
540 The RBC sample was gently pushed by hand into the filter [1]. An electric pump then flushed  
541 the rinsing medium across the filter [2]. In the microplate format, microfiltration was  
542 operated by vacuum aspiration in 2 steps; the sample was aspirated into the microsphere layer  
543 [1], and then rinsed using microfiltration medium [2]. The use of deep-well microplates  
544 allowed rinsing to be performed in a single step from the filtering to the receiver plate. (C)  
545 The microplate format displayed physiologically relevant retention (mean retention  $\pm$  SD) of  
546 an array of RBC subpopulations, including normal (nRBC), heat-stiffened RBC (HRBC), or  
547 RBC infected with *P. falciparum* asexual stages (Rings, Troph=Trophozoits,  
548 Schiz=Schizonts). (D) Microplate-based microfiltration is an open system preserving the  
549 morphology of infected and uninfected RBC (Giemsa-stained smears up- and down-stream  
550 from filters).

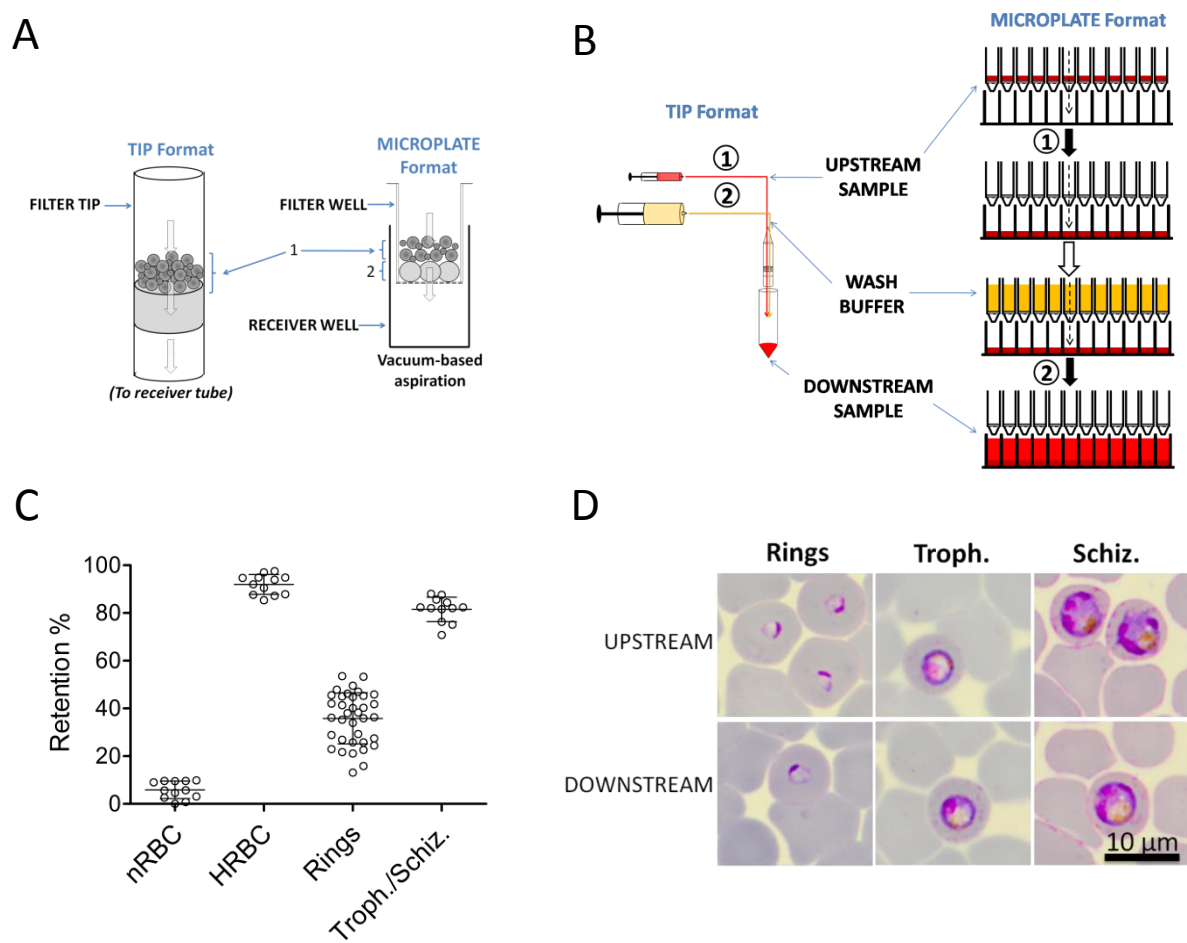
551 **Figure 2: Accurate, automated quantification of asexual and sexual stages up- and**  
552 **down-stream from filters.** A linear correlation was observed between parasitemia (A) and  
553 retention rates (B) determined either by Flow cytometry or by reference method (conventional  
554 counting on Giemsa-stained smears under the microscope). Parasite populations were filtered  
555 at different stages of asexual maturation under different experimental conditions to cover a

556 broad range of retention rates. Pooled results from 6 independent experiments are shown. (C)  
557 A linear correlation was observed between gametocyte concentrations determined either by  
558 Opera high content imaging or by reference method (hemocytometer) (n=3). (D) Example  
559 image taken using Opera confocal microscope of GFP-expressing Pf16 stage V gametocytes  
560 which were quantified and analyzed automatically.

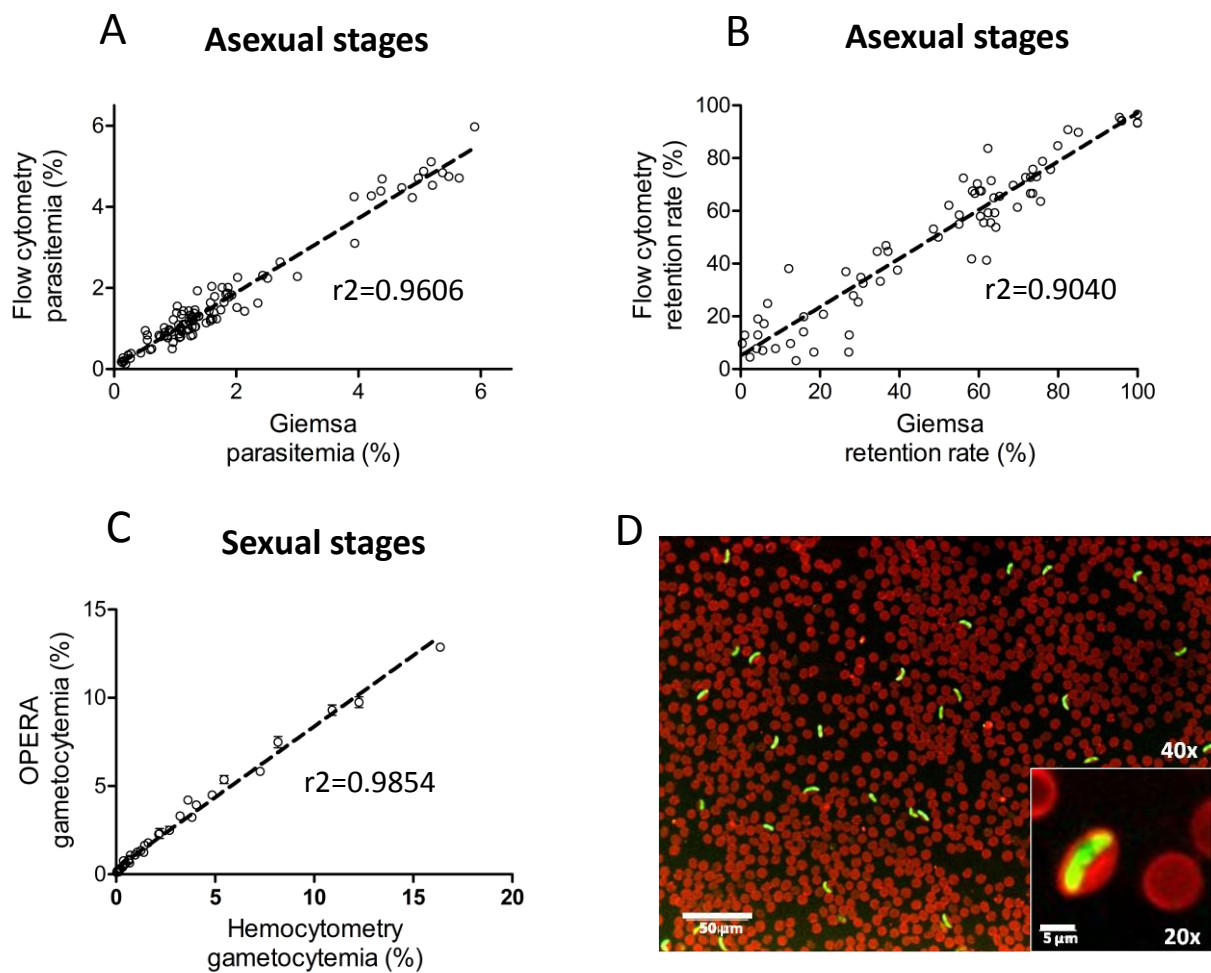
561 **Figure 3: Robust determination of retention rates after exposure of gametocytes to 40**  
562 **different compounds as a proof-of-concept for application for screening.** (A-i/) Single-  
563 well retention rates (mean values  $\pm$  SD) of mature stage V gametocytes were determined by  
564 microplate-based microfiltration after a 2- or 24-hour exposure to DMSO at 0.4% or  
565 calyculin (50 nM). Compared to unexposed controls (GAM V), 0.4% DMSO did not alter  
566 gametocyte retention rates (GAM V-DMSO, 2 & 24H) while calyculin significantly increased  
567 their mechanical retention (GAM calyculin, 2 & 24H). (A-ii/) The same concentration of  
568 calyculin (50 nM) did not significantly affect the viability of mature gametocytes at 24H. (B)  
569 Retention rates (mean values  $\pm$  SEM) of stage V gametocytes measured after a 24H exposure  
570 with 40 pharmacological compounds at 0.1 and 1  $\mu$ M (n=3). Only artemether, dihydro-  
571 artemisinin (DHA), artesunate (ART) artemisinin and artemisone (all endoperoxides)  
572 significantly decreased gametocyte ability to cross microsphere filters at 1 $\mu$ M (C, D).

573 **Figure 4: Mechanical clearance of compound-exposed mature gametocytes *in vitro* and**  
574 ***in vivo* to validate screening.** A) Mechanical retention (mean values  $\pm$  SD) of mature  
575 gametocytes in the filtering units of spleen-like biomimetic microchips as quantified by  
576 fluorescence microscopy. Calyculin-stiffened gametocytes were significantly enriched  
577 compared to controls. B) C57BL6 mice were treated (Full line) or not (Dotted line) with  
578 clodronate liposome for macrophage depletion prior to the transfusion of human RBC. Heat-  
579 stiffened human RBC (HRBC, Purple line) or control unheated RBC (nRBC, Red line) were

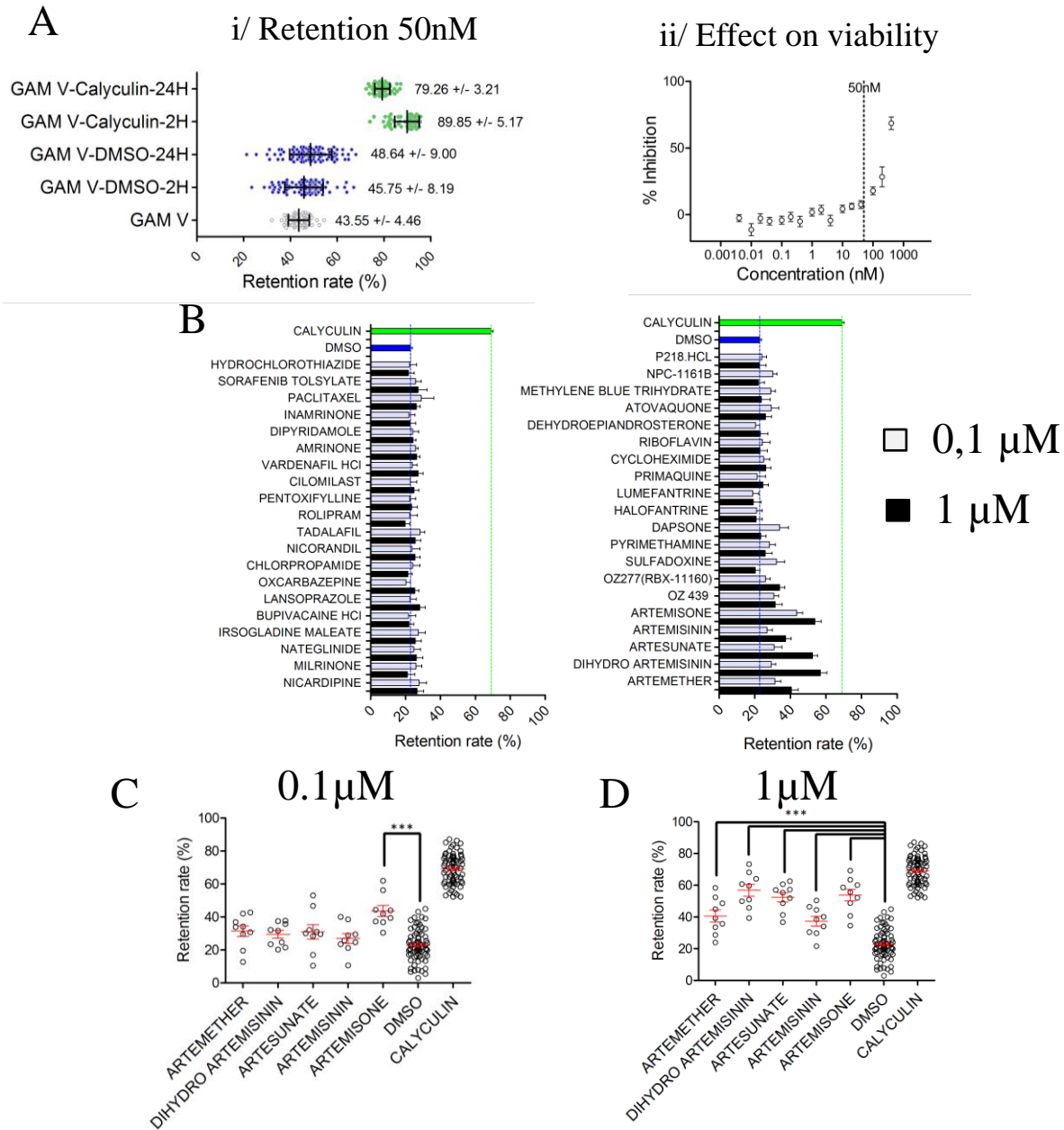
580 injected retro-orbitally after differential labeling with PKH-26 and PKH-67), for subsequent  
581 quantification using flow cytometry. Blood samples were collected at the tail vein 1, 90 and  
582 180 minutes post-injection (4 independent experiments for a total of 15 mice). C)  
583 Macrophage-depleted C57BL6 mice were transfused with human RBC infected with either  
584 immature (Dotted line, [stage I-IV]) or mature (Full line, [stage V]) gametocytes. The  
585 concentration of circulating gametocytes was determined on Giemsa-stained smears showing  
586 the persistence in circulation of a majority of RBC infected with mature gametocytes and the  
587 clearance of a majority of RBC infected with immature gametocytes (4 independent  
588 experiments for a total of 12 mice). D) Macrophage-depleted C57BL6 mice were transfused  
589 with human RBC infected with mature *P. falciparum* gametocytes, exposed just before  
590 transfusion either to calyculin at 100 nM or control DMSO 0.4% during 2 hours (2  
591 independent experiments for a total of 8 mice). A majority of mature gametocytes exposed to  
592 calyculin (Green line) were cleared while a majority of mature gametocytes exposed to the  
593 solvent (Blue line) stayed in circulation ( $p = 0.0286$ ). Red dotted line: nRBC and Purple  
594 dotted line: HRBC. Results from these mice experiments are expressed as mean values  $\pm$   
595 SEM.



**Figure 1: From the hand-made, single-sample prototype to a high throughput microfiltration assay using microplates.** (A) In the prototype, 5 – 25  $\mu\text{m}$  microspheres were directly deposited by hand above the anti-aerosol filter of a tip. In the microplate assay, 25 to 45 $\mu\text{m}$ -diameter microspheres [2] were poured using automated dispenser into 96-well filter mesh plates to support a 1.15 mm-thick layer of 5-15 $\mu\text{m}$  microspheres [1] forming the matrix mimicking the geometry of splenic slits [28]. (B) The driving force for microfiltration in the Tip format was applied using syringes connected to hermetic tubing. The RBC sample was gently pushed by hand into the filter [1]. An electric pump then flushed the rinsing medium across the filter [2]. In the microplate format, microfiltration was operated by vacuum aspiration in 2 steps; the sample was aspirated into the microsphere layer [1], and then rinsed using microfiltration medium [2]. The use of deep-well microplates allowed rinsing to be performed in a single step from the filtering to the receiver plate. (C) The microplate format displayed physiologically relevant retention (mean retention  $\pm$  SD) of an array of RBC subpopulations, including normal (nRBC), heat-stiffened RBC (HRBC), or RBC infected with *P. falciparum* asexual stages (Rings, Troph=Trophozoites, Schiz=Schizonts). (D) Microplate-based microfiltration is an open system preserving the morphology of infected and uninfected RBC (Giemsa-stained smears up- and down-stream from filters).

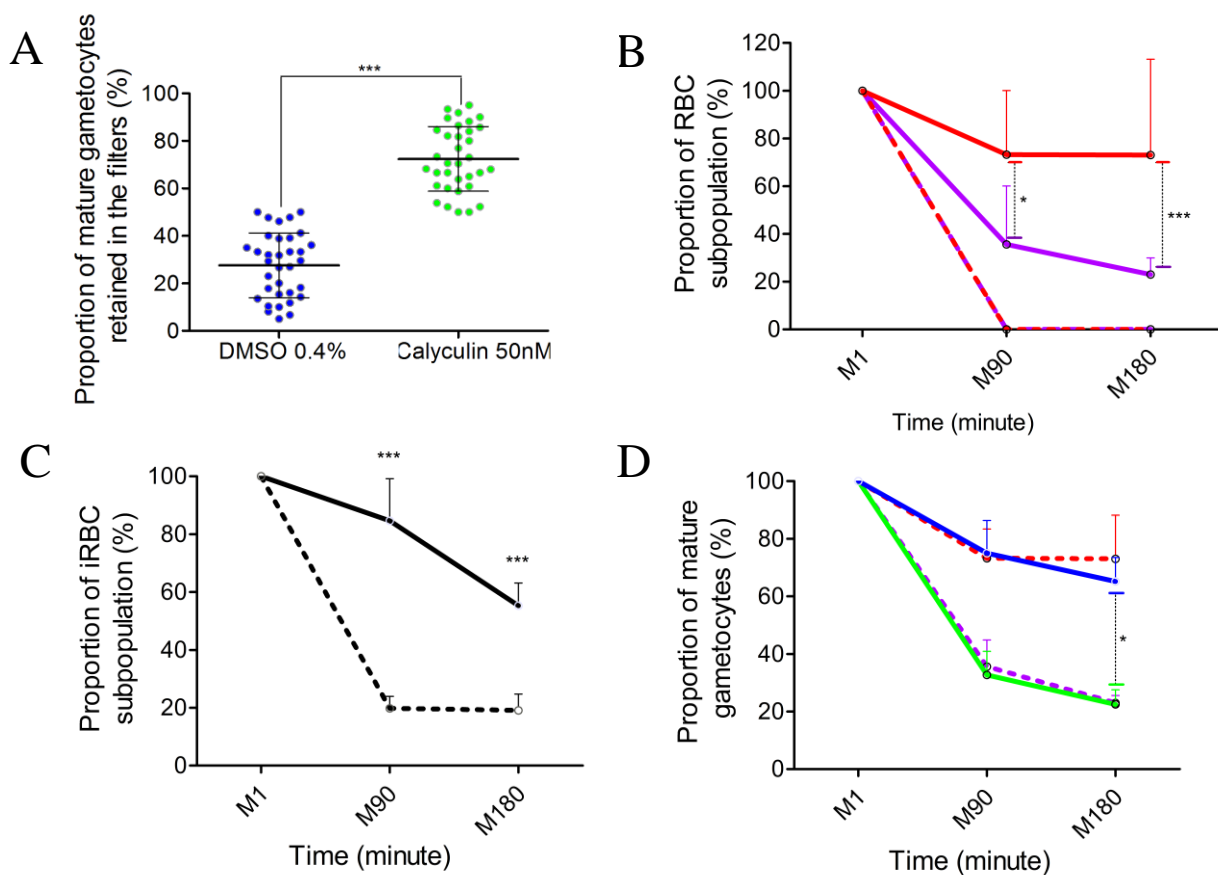


**Figure 2: Accurate, automated quantification of asexual and sexual stages up- and down-stream from filters.** A linear correlation was observed between parasitemia (A) and retention rates (B) determined either by Flow cytometry or by reference method (conventional counting on Giemsa-stained smears under the microscope). Parasite populations were filtered at different stages of asexual maturation under different experimental conditions to cover a broad range of retention rates. Pooled results from 6 independent experiments are shown. (C) A linear correlation was observed between gametocyte concentrations determined either by Opera high content imaging or by reference method (hemocytometer) ( $n=3$ ). (D) Example image taken using Opera confocal microscope of GFP-expressing Pf16 stage V gametocytes which were quantified and analyzed automatically.



**Figure 3: Robust determination of retention rates after exposure of gametocytes to 40 different compounds as a proof-of-concept for application for screening. (A-i/)** Single-well retention rates (mean values  $\pm$  SD) of mature stage V gametocytes were determined by microplate-based microspheruliferation after a 2- or 24-hour exposure to DMSO at 0.4% or calyculin (50 nM). Compared to unexposed controls (GAM V), 0.4% DMSO did not alter gametocyte retention rates (GAM V-DMSO, 2 & 24H) while calyculin significantly increased their mechanical retention (GAM calyculin, 2 & 24H). **(A-ii/)** The same concentration of calyculin (50 nM) did not significantly affect the viability of mature gametocytes at 24H. **(B)** Retention rates (mean values  $\pm$  SEM) of stage V gametocytes measured after a 24H exposure with 40 pharmacological compounds at 0.1 and 1  $\mu$ M (n=3). Only artemether, dihydro-artemisinin (DHA), artesunate (ART) artemisinin and artemisone (all endoperoxides) significantly decreased gametocyte ability to cross microsphere filters at 1  $\mu$ M **(C, D)**.





**Figure 4: Mechanical clearance of compound-exposed mature gametocytes in vitro and in vivo to validate screening.** A) Mechanical retention (mean values  $\pm$  SD) of mature gametocytes in the filtering units of spleen-like biomimetic microchips as quantified by fluorescence microscopy. Calyculin-stiffened gametocytes were significantly enriched compared to controls. B) C57BL6 mice were treated (Full line) or not (Dotted line) with clodronate liposome for macrophage depletion prior to the transfusion of human RBC. Heat-stiffened human RBC (HRBC, Purple line) or control unheated RBC (nRBC, Red line) were injected retro-orbitally after differential labeling with PKH-26 and PKH-67), for subsequent quantification using flow cytometry. Blood samples were collected at the tail vein 1, 90 and 180 minutes post-injection (4 independent experiments for a total of 15 mice). C) Macrophage-depleted C57BL6 mice were transfused with human RBC infected with either immature (Dotted line, [stage I-IV]) or mature (Full line, [stage V]) gametocytes. The concentration of circulating gametocytes was determined on Giemsa-stained smears showing the persistence in circulation of a majority of RBC infected with mature gametocytes and the clearance of a majority of RBC infected with immature gametocytes (4 independent experiments for a total of 12 mice). D) Macrophage-depleted C57BL6 mice were transfused with human RBC infected with mature *P. falciparum* gametocytes, exposed just before transfusion either to calyculin at 100 nM or control DMSO 0.4% during 2 hours (2 independent experiments for a total of 8 mice). A majority of mature gametocytes exposed to calyculin (Green line) were cleared while a majority of mature gametocytes exposed to the solvent (Blue line) stayed in circulation ( $p = 0.0286$ ). Red dotted line: nRBC and Purple dotted line: HRBC. Results from these mice experiments are expressed as mean values  $\pm$  SEM.