High-throughput microsphiltration to assess red blood cell deformability and screen for malaria transmission–blocking drugs

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The mechanical retention of rigid erythrocytes in the spleen is central in major hematological diseases such as hereditary spherocytosis, sickle-cell disease and malaria. Here, we describe the use of microsphiltration (microsphere filtration) to assess erythrocyte deformability in hundreds to thousands of samples in parallel, by filtering them through microsphere layers in 384-well plates adapted for the discovery of compounds that stiffen Plasmodium falciparum gametocytes, with the aim of interrupting malaria transmission. Compound-exposed gametocytes are loaded into microsphiltration plates, filtered and then transferred to imaging plates for analysis. High-content imaging detects viable gametocytes upstream and downstream from filters and quantifies spleen-like retention. This screening assay takes 3–4 d. Unlike currently available methods used to assess red blood cell (RBC) deformability, microsphiltration enables high-throughput pharmacological screening (tens of thousands of compounds tested in a matter of months) and involves a cell mechanical challenge that induces a physiologically relevant dumbbell-shape deformation. It therefore directly assesses the ability of RBCs to cross inter-endothelial splenic slits in vivo. This protocol has potential applications in quality control for transfusion and in determination of phenotypic markers of erythrocytes in hematological diseases.

Comparison with other methods

Microsphiltration is a dynamic method enabling the analysis of homogeneous cell populations (such as RBC concentrates) or specific cell subsets in a mixture of different RBC populations (e.g., RBCs infected with P. falciparum). Quantification of RBC subpopulations of interest upstream and downstream of filters is performed by flow cytometry or bright-field or fluorescence microscopy. Microsphiltration differs from membrane filtration in the geometry and relevance of the filtering gaps, which directly impact the deformation pattern displayed by filtered cells (generally, filtered cells with a cylindrical shape are observed with membrane filters and dumbbell-shaped filtered cells are observed with spleen-like microsphere filters). Micropipette aspiration, optical tweezers or ektacytometry generally measure RBC elongation or elasticity, and sometimes requires complex algorithms to analyze results. By contrast, microsphiltration simply...
Figure 1 | Schematic overview of the microsphiltration assay. (a,b) Calibrated microspheres are automatically loaded into 384-well frits-bottom filtration plates (a), and the resulting microsphiltration plates are then processed for storage and deferred use (b). (c,d) Mature Plasmodium falciparum gametocytes are produced in vitro and concentrated in fresh red blood cells (RBCs) (c) to further run the high-throughput microsphiltration screening assay (d). iRBC, gametocyte-infected red blood cell.

Quantifies a retention rate as a direct surrogate for cell ability to cross inter-endothelial splenic slits. Recently, using microfluidic chips, studies have mimicked the splenic retention of RBCs. However, current methods to assess cell deformability, including microfluidic technology, do not enable the analysis of more than a few cell samples per experiment and have not been amenable to high-throughput screening. Microsphiltration is relevant to exploring the ability of cell types to stay in circulation despite exposure to splenic mechanical filtration. By shifting from handmade filtering tips to 96- or 384-well microplates—into which microspheres are deposited by an automated dispenser—we have optimized the robustness and cost-effectiveness to meet the constraints of high-throughput pharmacological screening.

**Limitations**

Gametocyte production in vitro is time- and resource-consuming and requires training. Experience in laboratory automation is required to implement microsphiltration with a very high number of samples (Experimental design).

**Applications and potential extensions of the method**

Microsphiltration screening can potentially be used to identify compounds inducing the retention of circulating asexual stages of *P. falciparum* (ring stage; **Supplementary Fig. 1**) and can also be easily adapted to quantify the deformability of different circulating cell types. High-throughput capability is essential to observing small phenotypic differences in large populations of healthy subjects or patients, in order to better understand the biology of specific cell subpopulations and develop adapted therapeutic research. Thus, microplate microsphiltration potentially offers new pharmaceutical and therapeutic perspectives in regard to an interesting panel of disease states that involve abnormal cell deformability phenotypes. It has been used to study the impact of the sickle-cell trait on the course of malaria. Microsphiltration may also offer new immediate applications in the field of transfusion, especially for improving current processes used to store RBCs in blood centers, by enabling the simultaneous study of large blood collections during the first hours after blood draw. Using microplate microsphiltration for high-throughput probing of RBC units may also provide a new quality control to predict their transfusion yield, as morphological alterations related to deformability defects have recently been uncovered. Adaptation of the method to discover new classes of anticancer agents is conceivable because circulating cancer cells—especially stem-cell-like cancer cells—must be deformable in order to leave the primary tumor site, circulate and form distant metastatic lesions. Furthermore, microplate microsphiltration may facilitate investigations focusing on leukocytes, as their deformability is reduced in patients with trauma, sepsis or inflammatory diseases, negatively affecting the rheological properties of whole blood.

**Experimental design**

**Overview of the procedure.** Microsphere-based filters in 384-well microplates are made of two superimposed layers of 25- to 45- and 5- to 15-µm microspheres. Microspheres are loaded in suspension in an aqueous medium (microsphere suspension medium) using an automated dispenser (**Fig. 1a**; Steps 1–7). Microsphere-loaded plates are dried overnight on a slide warmer in a compartment ventilated with dry airflow, then packed in bags conditioned for storage, transport and deferred use.
**PROTOCOL**

**Figure 2** | High-content imaging to count gametocyte-infected RBCs before and after microsphiltration, and to quantify retention rates. The image was generated using the ×60 objective of an Opera high-resolution disc imager. RBC membranes were stained with CellMask Orange. Gametocytes were discriminated from RBCs based on DNA staining with Syto 40 Blue (green color) and mitochondrion staining with Mitotracker Deep Red FM (blue color). Inset—magnification of one gametocyte-infected RBC.

(Fig. 1b; Step 8). For the screening application described here, *P. falciparum* NF54 sexual transmission stages (mature gametocytes) are produced as described. Mature gametocytes are then concentrated (using density-gradient centrifugation (Nycoprep)) at a final 3–5% gametocytemia level in fresh, uninfected RBCs (Fig. 1c; Steps 9–20). Forty-five microliters of gametocyte suspension at hematocrit 0.5% is transferred to a 384-well plate, where it is exposed to pharmacological compounds for 2 or 24 h (Fig. 1d, Steps 21–24). Using an automated dispenser, compound-exposed gametocytes are resuspended and transferred as 25- to 15-µm spheres that are imaged using a high-resolution spinning disc confocal microscope (Opera; Fig. 2 and Supplementary Fig. 2, Steps 36–38). The filtered samples (Ds = downstream from filters) and their respective controls (Up = upstream from filters; unfiltered gametocyte populations remaining in compound plates) are then automatically transferred (~35,000 cells per well) to separate imaging plates prefilled with 30 µl of staining solution (Steps 25, 34, 35 and 39–40). After overnight incubation at 24 °C, the stained gametocyte-infected RBC samples form monolayers that are imaged using a high-resolution spinning disc confocal microscope (Opera; Supplementary Fig. 2). Comparison of imaged samples upstream and downstream of filters enables specific quantification of gametocyte retention rates independently of killing.

**Automated sample handling.** Although manual loading of microspheres and handling of samples during microsphiltration can be performed by well-trained personnel, in the assay described here, Biomek NX™ liquid handlers equipped with 384-channel pipetting heads and vacuum-filtration devices are used to maximize reproducibility of both microsphere-layer loading into frits-bottom filtration plates and gametocyte microsphiltration. To maximize the assay throughput and comply with the functional compartmentalization of our laboratory, two Biomek NX™ dispensers plus two Biomek vacuum-filtration devices were used to prepare microsphiltration plates and run microsphiltration in an independent manner. Important parameters for automated handling steps involved in this protocol are summarized in Tables 1 and 2.

**Optimization.** This protocol describes an updated version of our previously published 96-well plate microsphiltration assay, streamlined to increase its throughput and selectivity. A dispenser-assisted method has been developed to prepare 384-well microsphiltration plates, opening the way to the screening of large chemical collections.

In the former version of the assay, the retention of mature gametocytes was quantified from imaged monolayers of elongated GFP-expressing gametocytes within a RBC population stained with CellMask Orange. However, a proportion of gametocytes killed by a pharmacological agent can still remain elongated and crescent shaped, excluding morphology as a reliable criterion for assessing gametocyte viability. From a screening perspective, this implies that the hits selected with the previous assay readout had to be subsequently tested in an orthogonal assay in order to exclude false positives characterized by gametocytocidal activity, which can substantially affect retention rates. Here, the nucleic acid stain Syto 40 Blue and the mitochondrial probe Mitotracker Deep Red FM are used in combination to quantify the percentage of elongated, crescent-shaped gametocytes with preserved mitochondrial potential within RBCs stained with CellMask Orange. Thus, the gametocytocidal activity of compounds (referred as killing) and splenomimetic retention rates are measured in the same filtration experiment. Killing is determined by comparing the proportion of viable gametocytes (upstream of the filters) in compound versus control wells. Retention is measured by comparing the proportion of viable gametocytes upstream and downstream of the filters.

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**TABLE 1 | Automated microsphiltration plate preparation sequence using a Biomek NX™ dispenser.**

<table>
<thead>
<tr>
<th>Setting</th>
<th>Dispenser function and direction</th>
<th>Handling parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 4 (pre-fill with PBS-A)</td>
<td>Transfer: PBS-A reservoir → bottom-filter plate</td>
<td>• Asp-disp speed: 90 µl/s • Volume: 60 µl</td>
</tr>
<tr>
<td>Step 5 (transfer 25- to 45-µm spheres)</td>
<td>Transfer: spheres reservoir → bottom-filter plate</td>
<td>• Asp speed: 10 µl/s • Disp. speed: 90 µl/s • Volume: 15 µl</td>
</tr>
<tr>
<td>Tip wash</td>
<td>Mix: tip wash reservoirs (H2O)</td>
<td>• Asp-disp speed: 90 µl/s • Repetition: 5×</td>
</tr>
<tr>
<td>Step 6 (transfer 5- to 15-µm spheres)</td>
<td>Transfer: spheres reservoir → bottom-filter plate</td>
<td>• Asp speed: 10 µl/s • Disp. speed: 90 µl/s • Volume: 12 µl</td>
</tr>
<tr>
<td>Tip wash</td>
<td>Mix: tip wash reservoirs (H2O)</td>
<td>• Asp-disp speed: 75 µl/s • Repetition: 5×</td>
</tr>
</tbody>
</table>

Asp, aspirate; Disp, dispense.
### Automated microsphiltration assay implementation sequence using a Biomek NX™ dispenser.

<table>
<thead>
<tr>
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| Step 27 (re-humidify microsphiltration plate) | Transfer: culture medium reservoir → microsphiltration plate | • Asp speed: 90 µl/s  
  • Disp. speed: 50 µl/s  
  • Volume: 50 µl |
| Step 29 (prefill sterile reservoir plate with PBS-A) | Transfer: culture medium reservoir → reservoir plate | • Asp speed: 90 µl/s  
  • Disp. speed: 50 µl/s  
  • Volume: 25 µl |
| Step 31 (prefill microsphiltration plate with culture medium) | Transfer: culture medium reservoir → microsphiltration plate | • Asp speed: 90 µl/s  
  • Disp. speed: 50 µl/s  
  • Volume: 25 µl |
| Step 32 (resuspend compound-exposed gametocytes) | Mix: gametocyte-compound plate | • Asp-disp speed: 90 µl/s  
  • Volume: 20 µl  
  • Repetition: 1× for each well quadrant and the well center  
  • Distance tips—RBC layer: 1 mm |
| Step 33 (transfer gametocytes to microsphiltration plate) | Transfer: gametocyte-compound plate → microsphiltration plate | • Asp speed: 90 µl/s  
  • Disp. speed: 50 µl/s  
  • Volume: 25 µl |
| Tip wash | Mix: tip wash reservoirs (H₂O) | • Asp-disp speed: 75 µl/s  
  • Repetition: 5× |
| Step 34 (dilute upstream gametocyte sample) | Transfer and mix: culture medium reservoir → gametocyte-compound plate | • Asp speed: 90 µl/s  
  • Disp. speed: 50 µl/s  
  • Volume: 80 µl  
  • Mix repetition: 3× (20 µl) |
| Step 35 (transfer upstream gametocyte imaging plate) | Transfer and mix: gametocyte-compound plate → upstream imaging plate | • Asp speed: 25 µl/s  
  • Disp. speed: 10 µl/s  
  • Volume: 12.5 µl  
  • Mix repetition: 3× (15 µl) |
| Tip wash | Mix: tip wash reservoirs (H₂O) | • Asp-disp speed: 75 µl/s  
  • Repetition: 5× |
| Step 38 (rinse microsphiltration plate) | Transfer: culture medium reservoir → microsphiltration plate | • Asp speed: 90 µl/s  
  • Disp. speed: 50 µl/s  
  • Volume: 80 µl |
| Step 39 (transfer downstream gametocytes to downstream imaging plate) | Transfer and mix: reservoir plate → downstream imaging plate | • Asp speed: 25 µl/s  
  • Disp. speed: 10 µl/s  
  • Volume: 12.5 µl  
  • Mix repetition: 3× (15 µl) |

Asp, aspirate; Disp, dispense.

In addition, a storage process was developed to enable the shipment and deferred use of microsphiltration plates, which should facilitate the diffusion of the method.

### Assay controls.

A set of RBC controls (Fig. 3) have been defined to calibrate and monitor the performances of the microsphiltration assay. For calibration, heat-stiffened28 or glutaraldehyde-fixed RBCs40 should be retained at >85% in microsphere-based filters. Normal RBCs used to dilute glutaraldehyde- or heat-stiffened RBCs provide a negative control, the retention of which should be <10%. RBCs hosting asexual stages of *P. falciparum* are also routinely used as a positive control, as their retention gradually increases with their progression through the parasite cycle10,28. Moreover, their use speeds up the optimization process, as their culture is faster and easier than that of gametocytes.

To monitor the performance of filtration during screening, mature gametocytes exposed to 50 nM calyculin A28 5 µM and cipargamin (NITD609, KAE609)41 are used as positive retention controls to define the hit retention threshold and the highest retention rate of the assay, respectively. Calyculin A specifically stiffens mature gametocytes independently of killing16,28. Assessing killing activities involves the use of 50 µM Gentian Violet38 as positive control. Finally, mature gametocytes exposed to 0.05% (vol/vol) DMSO are used as a negative control to normalize both retention and killing values.

### Parameters affecting the setup of the microsphiltration assay.

The following experimental parameters that affect the outcome of microsphiltration should be considered when preparing microsphiltration plates, as described further below: (i) the properties of the microplate bottom filter mesh/frits, the thickness of the microsphere layers and the distribution of the microsphere sizes in a commercial batch, (ii) the composition of the suspension medium for RBCs and gametocytes and the synchronicity of the parasite culture, (iii) the amount of rigid RBCs to filter, as determined by saturation limits. A successful and reproducible setup for the assay requires a series of preliminary tests to finely calibrate these parameters due to differences observed from one microsphere batch to another, as well as to variations in serum and RBC sources.

(i) **Bottom filter mesh, microsphere layer thickness and microsphere size distribution.** Microsphere layers form a network of narrow channels corresponding to intersphere spaces of uneven widths and lengths. The narrowest spaces, created when two or three of the smallest (5-µm) microspheres are adjacent, are ~2 µm wide and 2 µm long. These impose a deformability challenge on RBCs flowing through that is reminiscent of the challenge induced by inter-endothelial slits in the human spleen. Because microsphere layers contain a mixture of microsphere sizes, the width of the intersphere spaces is predicted to fluctuate between 1.85 and 5.67, and 9.25 and 16.7 µm, for the 5- to 15-µm and the 25- to 45-µm microsphere layers, respectively. Thus, the 25- to 45-µm microsphere layer does not contribute to the mechanical retention of RBCs, as its smallest intersphere spaces are larger than the diameter of a RBC (RBC diameter ~6–9 µm). This 25- to 45-µm microsphere layer is necessary to prevent the smaller 5- to 15-µm microspheres from leaking across the microplate bottom frits (the pore size of which is 20 µm). Frits-bottom filtration plates used for microsphiltration have been selected based on their frit pore size (>15 and <25 µm) and stability.

In addition, a storage process was developed to enable the shipment and deferred use of microsphiltration plates, which should facilitate the diffusion of the method.

### Protocols.

#### Automated microsphiltration assay implementation sequence using a Biomek NX™ dispenser.

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  • Mix repetition: 3× (20 µl) |
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  • Volume: 12.5 µl  
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| Step 39 (transfer downstream gametocytes to downstream imaging plate) | Transfer and mix: reservoir plate → downstream imaging plate | • Asp speed: 25 µl/s  
  • Disp. speed: 10 µl/s  
  • Volume: 12.5 µl  
  • Mix repetition: 3× (15 µl) |

Asp, aspirate; Disp, dispense.
upon exposure to chemicals used to solubilize the pharmacological compounds to be tested. Harvard Apparatus 96-well filter-bottom plates and Seahorse 384-well frits-bottom plates have been selected and validated for use in microfiltration. The thickness and composition of the microsphere layers have been selected by a trial-and-error approach, with retention rates of control RBC populations used as the validation output. Multiple batches of 5- to 15-µm AmTech microspheres or different mixtures of 5- to 15-µm and 15- to 25-µm IPS (Industrie des Poudres Sphériques) microspheres in 50:50 to 30:70 proportions have been validated for use in microfiltration. Using the same batch of microspheres across experiments is important for reproducibility of results. Microfiltration delivers optimal results when day 12–14 and day 16–19 mature gametocytes are produced using Avery’s42 and Delves’38 protocols, respectively.

(iii) Saturation limits for microsphere filters. To prevent unspecific clogging of filters, the mature gametocyte culture should be purified before microfiltration. Density-gradient (Nycodenz) centrifugation has been selected as the most efficient method to separate mature gametocytes from cell debris, parasite remnants, pyknotics and spherocytes created by the long culture process. Gametocyte purification is followed by dilution with fresh uninfected RBCs at 2–5% gametocytemia final concentration, hematocrit = 0.5%. When using microfiltration to study uninfected RBCs, the population of interest should be labeled with PKH10 or CFSE38 and diluted in unlabeled control RBCs in a proportion of 2–5% of test-labeled RBCs in 95–98% unlabeled control RBCs. Alternatively, control RBCs (still 95–98% of the whole) can be labeled with PKH or CFSE, and the population of interest left unlabeled. This second approach considerably reduces the time needed for labeling (as there is only a single large sample, rather than many small samples, to label) and limits the intervention to the RBC population of interest. Control RBCs used to dilute test RBCs should be kept in preservation medium (such as SAGM (saline, adenine, glucose, mannitol)) for a maximum of 15 d before use for microfiltration.

Figure 3 | Controls. (a) Retention rates of control RBC populations used to calibrate the performances of microsphere-based filters. Retention rates from two independent experiments in which CFSE-stained uninfected RBCs (HRBCs and Gluta-fixed RBCs) or Sybr Green-stained P. falciparum-infected RBCs (R 2–8 h and T/S 35–41 h lanes) were quantified using flow cytometry against ≥20,000 events (Supplementary Figs. 7 and 8). (b,c) Microfiltration screening assay controls. Retention rates of mature gametocytes (b) exposed during 2 or 24 h to 0.05% (vol/vol) DMSO (negative control), 50 nM calyculin A (CAL; positive control for gametocyte stiffening) or 2.5 µM cipargamin (NITD609; positive control for gametocyte swelling–induced stiffening). (c) Killing rates of mature gametocytes exposed to either 0.05% (vol/vol) DMSO (negative control) or 50 µM Gentian Violet (G. Violet; positive control) for 2 or 24 h. Gluta-fixed RBCs, glutaraldehyde-fixed, stiff RBCs; HRBCs, heat-stiffened RBCs; RBCs 1–2 weeks, RBCs used to dilute the gametocytes or the uninfected RBCs to test; R 2–8 h, young P. falciparum at asexual ring stages; T/S 35–41 h, P. falciparum at asexual stages (trophozoites and schizonts).
Valladolid, Spain. **CAUTION** Human blood is screened for the presence of human pathogens. It must be handled in agreement with institutional and governmental regulations while using personal protective equipment and approved biological waste disposal. The human biological samples were sourced ethically, and their research use was in accord with the terms of the informed consents.

- RPMI 1640 medium with 25 mM HEPES (without L-Glutamine; Sigma-Aldrich, cat. no. R8866)
- Sodium bicarbonate (HNaCO\(_3\); Merck, cat. no. S7671)
- Hypoxantine powder (Sigma-Aldrich, cat. no. H9377)
- L-Glutamine (Merck, cat. no. 100289)
- (+)-Glucose (Sigma, cat. no. G8270)
- Albumax II (Life Technologies, cat. no. 11021-037)
- PBS (Sigma, cat. no. P4417)
- Ethanol (70% vol/vol), DAB pure (tested to conform to the product specifications of the Deutsche Artensie Buch), Pharma grade; PanReac Appli-Chem, cat. no. A09113-5000) **CAUTION** Ethanol is a volatile and flammable liquid that must be handled while wearing personal protective equipment in accordance with government regulations.
- Nycoprep 1.077 (axis-shield density-gradient medium; Alere Technologies, cat. no. Z654663-1EA)
- PBS (Sigma, cat. no. 1114550)
- Glutaraldehyde solution (Merck, cat. no. G4004) **CAUTION** Glutaraldehyde is a toxic and strongly irritating liquid that must be handled while wearing personal protective equipment.
- Giemsa’s Azur Eosin Methylene Blue solution (Merck, cat. no. 1092040100) **CAUTION** This solution is a combustible liquid with high toxicity that must be handled while wearing personal protective equipment in accordance with government regulations.
- Methanol (GPR Rectapur; VWR, cat. no. 20846.361) **CAUTION** Methanol is flammable and toxic. It must be handled with care and in accordance with governmental regulations while wearing personal protective equipment.
- Syto 40 Blue fluorescent nucleic acid stain (Thermo Fisher Scientific, cat. no. S11351) **CRITICAL** Other nucleic acid stains can be used, such as Sybr Green, but these would require adjustment of the imaging parameters.
- Sybr Green I nucleic acid gel stain (Thermo Fisher Scientific, cat. no. S7567)
- CellMask Orange plasma membrane stain (Thermo Fisher Scientific, cat. no. C10045)
- CellTrace CFSE Cell Proliferation Kit (Thermo Fisher Scientific, cat. no. C34554)
- PKH 67 (Merck, cat. no. PK67G-1K1)
- Pyrex borosilicate glass tube (Fisher Scientific, cat. no. 10422205)
- DMSO (299.5% vol/vol) (Sigma, cat. no. D2650)
- Calycin A from Discordia clara (Thermo Fisher Scientific, cat. no. C5552)
- Gentian Violet (MolPort, cat. no. 002-133-551)
- Cipargamin (Angen, CAS no. 1193134-23-6; product no. AG0001MA/AGN-PC-06D6LA)
- **CAUTION** Complete gametocyte culture medium is referred to as gametocyte culture medium in this protocol.
- **CAUTION** Sterility of culture reagents should be preserved by working in a class II microbiological safety cabinet or ensured by filtration with a 0.2-µm filter Stericup. Microspheres can be purchased from alternative providers.
- **CAUTION** Soldering irons are harmful upon contact with skin. Handle it with care and following approval from laboratory institution.
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- 384-Well frits-bottom filtration plates (Seahorse Biosciences, cat. no. SEA201035100)
- Eppendorf polypropylene conical-bottom 384-deep-well plates (Fisher Scientific, cat. no. 10581874)
- Electrical hair dryer (1,750 W) (UFESA, model no. SC8380)
- Soldering iron (230 V, 40 W) (Screwfix, cat. no. 40326)
- 384-Well clear polystyrene microplates (Greiner Bio-One, cat. no. 781101)
- Opera Phenix high-content screening system (PerkinElmer, cat. no. HH114000000)
- Collagen-coated Cellcarrier-384 ultra microplates (PerkinElmer, cat. no. 6057708)
- Clear adhesive seal for microplates (PerkinElmer, cat. no. 6050185)
- Greiner microplate lid (Merck, cat. no. L3411-100EA)

**REAGENT SETUP**

- Eppendorf Safe-Lock microcentrifuge tubes (Merck, cat. no. T9661-1000EA)
- Olympus binocular microscope (Olympus Life Science, model no. CX31)
- Neubauer chamber (Brand, cat. no. 717810)
- Microscope slides (Marienfeld, cat. no. 1000912)
- Microscope slide-staining jar (Ted Pella, cat. no. 432-1)
- Sartorius eLINE multichannel electronic pipettor (Merck, cat. no. Z654663-1EA)
- Slide warmer (LabScientific, model no. XH-2001)
- Qb dry block heating system (Grant, model no. QBD2)
- Water bath (Julabo, model no. PURA 10, cat. no. 9550410)
- Hercass 240i CO\(_2\) incubators (Thermo Scientific, cat. no. S1026884)
- Biomek filtration system with vacuum valve unit (Beckman Coulter) including the vacuum filtration manifold base (cat. no. 609670), square well collar (cat. no. 609673) and tubing kit (cat. no. 609676)
- Electric vacuum pump (220 V/50 Hz) (Millipore, cat. no. WP6122050)
- Filtration unit (e.g., Millex, cat. no. SLFA05000)
- Vacuum gauge kit (Millipore, cat. no. SVMHS705)
- Moisture barrier bags (Protective Packaging, ref. no. PP005 JUMBO - 7444 LAB IW ID20x30 cm)
- 50-g silica gel sachets (Protective Packaging, ref. no. PSILGE25D - defence standard 2.5g silica-gel 400/bx)
- Lab scale balance (Mettler Toledo, model no. PJ3600)
- HP digital dispensor (HP, model no. D300e)
- HP D4 dispense-head cassettes (HP, cat. no. B3F33A)
- Biomek NX liquid-handling lab automation workstation (Beckman Coulter, cat. no. 989402) **CRITICAL** This dispensor requires a laboratory air-supply system. Alternative dispensor systems can be purchased from other equipment providers.
- Biomek AP384 P30 tips (Beckman Coulter, cat. no. 719222)
- Robotic reservoirs (Nalgene; Thermo Scientific, cat. no. 1200-1300)
- 3 M Breath–protecting mask FFPE (Lab Unlimited, cat. no. 4D1-6269582)
- Stericup-GP (0.22 µm, 500 ml) (Merck-Millipore, cat. no. SCGP053R)
- Test tube rotator (Labino, model no. LD79)
- 25–45 µl (type 3 solder powder-Sn96.5/Ag0.5, Invetec Performance Chemicals, AmTech, cat. no. 9030) or (IPS, ref. SAC305-3T-25/45-D73TN)
- 5–15-µl type 6 solder powder Sn96.5/Ag0.3 (Invetec Performance Chemicals; AmTech, cat. no. 9060) or (IPS, ref. nos. SAC305-6A-5/5-JD76AU and SAC305-5A-15/25-D75AU) **CRITICAL** Microspheres are irritating and must be handled while wearing appropriate personal protective equipment, including breathing-protecting mask and disposed of in an approved waste disposal system. **CRITICAL** Microspheres can be purchased from alternative providers.
- 384-Well frits-bottom filtration plates (Seahorse Biosciences, cat. no. SEA201035100)
- Eppendorf polypropylene conical-bottom 384-deep-well plates (Fisher Scientific, cat. no. 10581874)
- 384-Well clear polystyrene microplates (Greiner Bio-One, cat. no. 781101)
- Cipargamin (Angene, CAS no. 1193134-23-6; product no. AG0001MA/AGN-PC-06D6LA)
- **CAUTION** Sterility of culture reagents should be preserved by working in a class II microbiological safety cabinet or ensured by filtration with a 0.2-µm filter Stericup.

**Preparation of suspension medium for microspheres** Allow 10 g of Alumax II powder to dissolve over 2 h in 1 liter of sterile 1× PBS, using stir-bar agitation at a temperature <40 °C (to avoid Alumax polymerization). Filter-sterilize using a 0.2-µm filter Stericup and store as 500-ml bottles at 4 °C up to 8 weeks.

**Preparation of incomplete gametocyte culture medium** Five-liter batches of incomplete gametocyte culture medium are routinely prepared at a time for subsequent division into convenient 500-ml bottles, storable for up to 6 months at 4 °C. For that purpose, dissolve 250 mg of hypoxanthine in 5 liters of dH\(_2\)O in a canonical flask, using stir-bar agitation at room temperature (−18–24 °C) over 3 h. Then add 79.45 g of RPMI powder plus 10 g of NaHCO\(_3\) and allow these to dissolve for an extra hour. Filter-sterilize this incomplete gametocyte culture medium using a 0.2-µm filter Stericup for 500-ml volumes, which are storable for up to 1 year at −20 °C.

**Preparation of human serum** Five-liter batches of heat-inactivated human serum are routinely prepared at a time for subsequent division into 200-ml aliquots, storable for up to 1 year at −20 °C. To this aim, heat-inactivate human serum bags at 56 °C over 30 min, and then pool these in a 5-liter conical flask for subsequent division into 250-ml aliquots and freeze at 20 °C until use.

**Preparation of complete gametocyte culture medium** Complete gametocyte culture medium is referred to as gametocyte culture medium in this protocol.
**Preparation of glutaraldehyde-fixed RBCs**

To a 15-ml Falcon tube, add 500 µl of 1.60% (vol/vol) glutaraldehyde to 500 µl of packed CSFE-stained RBCs at hematocrit 10% in RPMI 1640 medium, then incubate for 10 min at room temperature, protected from light. Stop the fixation reaction by adding 10 ml of gametocyte culture medium and pelleting the RBCs by centrifugation at 1,000 × g for 5 min at 20 °C. Wash the RBC pellet three times by adding 10 ml of gametocyte culture medium and centrifuging at 1,000 × g for 5 min at 20 °C. Store for up to 1 week at hematocrit 2% in gametocyte culture medium, at 4 °C and protected from light.

**Preparation of heat-stiffened RBCs**

Leukocyte-reduced human blood at hematocrit 60% in SAGM is routinely aliquoted into 45-ml volumes in Falcon tubes and stored at 4 °C for up to 15 d before use. Before use, centrifuge at 2,000 × g, 4 °C, for 5 min. Then, remove the SAGM and wash with 20 ml of gametocyte culture medium by repeating the centrifugation. Store at hematocrit 50% in gametocyte culture medium at 4 °C for up to 5 d.

**Preparation of RBCs for gametocyte culture and dilution following Nycoprep isolation**

Leukocyte-reduced human blood at hematocrit 60% in RPMI 1640 medium, then incubate at room temperature for 20 min, protected from light. Stop the fixation reaction by adding 10 ml of gametocyte culture medium and pelleting the RBCs by centrifugation at 1,000 × g for 5 min at 20 °C. Wash the RBC pellet three times by adding 10 ml of gametocyte culture medium and centrifuging at 1,000 × g for 5 min at 20 °C. Store for up to 1 week at hematocrit 2% in gametocyte culture medium at 4 °C and protected from light.

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To prepare 4.5 liters of complete gametocyte culture medium, remove two serum bottles from the freezer, let them thaw over 1 h in a water bath set at 37 °C and split these into 45-ml volumes in Falcon tubes. Centrifuge for 20 min at 5,000 × g, 37 °C. Remove surface debris using a pipette and add 55 ml of serum to 500 ml of incomplete gametocyte culture medium. Filter-sterilize using a 0.2-µm filter Stericup and store at 4 °C for up to 10 d.

**Preparation of Mitotracker Deep Red FM stock**

Resuspend the 250 µg of Mitotracker powder in 460 µl of DMSO to a final concentration of 1 mM, divide into 50-µl aliquots and store at −80 °C, protected from light, for up to 6 months.

**Preparation of upstream and downstream staining solutions for imaging the layers of gametocyte-infected RBCs**

A 60-ml volume of both upstream and downstream gametocyte staining PBS solutions is routinely prepared at a time for each assay run of five plates. For the upstream staining solution, add 840 µl of Syto 40 Blue, 42 µl of Mitotracker Deep Red FM and 24 µl of CellMask Orange to 60 ml of sterile 1× PBS. Mix thoroughly and store at 4 °C, protected from light, for up to 24 h.

**Preparation of heat-stiffened RBCs**

Leukocyte-reduced human blood at hematocrit 60% in RPMI 1640 medium, then incubate for 20 min using a water bath. Wahs the RBC pellet twice using gametocyte culture medium by centrifugation at 1,000 × g, 4 °C, for 5 min. Then, remove the SAGM and wash with 20 ml of gametocyte culture medium by repeating the centrifugation. Store at hematocrit 50% in gametocyte culture medium at 4 °C for up to 5 d.

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**Preparation of heat-stiffened RBCs**

Leukocyte-reduced human blood at hematocrit 60% in RPMI 1640 medium, then incubate for 20 min using a water bath. Wash the RBC pellet twice using gametocyte culture medium by centrifugation at 1,000 × g, 4 °C, for 5 min. Then, remove the SAGM and wash with 20 ml of gametocyte culture medium by repeating the centrifugation. Store at hematocrit 50% in gametocyte culture medium at 4 °C for up to 5 d.

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**Preparation of RBCs for gametocyte culture and dilution following Nycoprep isolation**

Leukocyte-reduced human blood at hematocrit 60% in RPMI 1640 medium, then incubate at room temperature for 20 min, protected from light. Stop the fixation reaction by adding 10 ml of gametocyte culture medium and pelleting the RBCs by centrifugation at 1,000 × g for 5 min at 20 °C. Wash the RBC pellet three times by adding 10 ml of gametocyte culture medium and centrifuging at 1,000 × g for 5 min at 20 °C. Store for up to 1 week at hematocrit 2% in gametocyte culture medium, at 4 °C and protected from light.

**EQUIPMENT SETUP**

**Preparation and use of the Biomek vacuum-filtration device**

Component assembly follows general manufacturer’s operating instructions and is illustrated in Figure 4. Use the four-way tubing connector coupled to the vacuum gauge and vacuum valve to control a c

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**TABLE 3** | **Operahigh spinning disc confocal imager acquisition parameters.**

<table>
<thead>
<tr>
<th>Setting</th>
<th>Exposure 1 (Syto 40 Blue)</th>
<th>Exposure 2 (Mitotracker Deep Red FM)</th>
<th>Exposure 3 (CellMask Orange)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Focus height</td>
<td>0–1 µm</td>
<td>635 nm</td>
<td>532 nm</td>
</tr>
<tr>
<td>Laser</td>
<td>405 nm</td>
<td>635 nm</td>
<td>532 nm</td>
</tr>
<tr>
<td>Camera</td>
<td>No. 1—450/50 nm</td>
<td>No. 3—690/50 nm</td>
<td>No. 2—585/40 nm</td>
</tr>
<tr>
<td>Camera exposure time</td>
<td>200 ms</td>
<td>400 ms</td>
<td>650 ms</td>
</tr>
<tr>
<td>Detection dichroic mirror</td>
<td>568 nm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary dichroic mirror</td>
<td>405/532/635 nm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lens</td>
<td>×20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sublayout</td>
<td>Six fields</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Store for up to 1 week at hematocrit 2% in gametocyte culture medium, at 4 °C and protected from light.
water before first use is also recommended. Necessary labware and important parameters for automated processing of microspheres and gametocytes throughout the microsphiltration assay are summarized in Tables 1 and 2, respectively.

**Upstream and downstream imaging plates for use in gametocyte microsphiltration** Load 30 µl of upstream and 30 µl of downstream staining solutions into the wells of the upstream and downstream imaging plates, respectively. These plates can be prepared 1 d in advance and stored overnight at 4 °C protected from light.

**Opera high-resolution spinning disc confocal imager** In accordance with the detailed procedures communicated by the manufacturer (http://www.perkinelmer.com/lab-products-and-services/cellular-imaging/), check liquid levels before use and, if necessary, fill the water bottle reservoir with sterile reverse-osmosis water and empty the bin bottle. Select the plate type ‘Cell carrier 384 Ultra’ and the ×20 water objective. Flush the Opera micro-fluidic system. Switch on the 405-, 532- and 635-nm lasers, along with the 450/50-, 585/40- and 690/50-nm cameras. Set up exposure parameters as described in Table 3. (When setting up the imaging protocol for the first time, use the 96-well Opera adjustment plate to generate reference images and a skew analysis.) Before running the automated imaging protocol according to the manufacturer’s instructions, finely adjust both light source power and exposure time using an upstream imaging plate (from Step 40) loaded with the fluorescent cell samples to image. Optimal exposure parameters should yield unsaturated fluorescent images visually (Fig. 2: Image analysis and controls section). Optimal imaging conditions should yield gametocytemia levels that are similar to those previously quantified using control Giemsa-stained films (PROCEDURE, Step 19). Save the exposure parameters. Finally, create both plate layout and well sublayout files.

**Image analysis and controls** Image analysis of gametocyte-infected RBC monolayers from upstream and downstream imaging plates uses the Columbus image data storage and analysis system, which is coupled to the Opera imager. Optimization of the image analysis script involves the use of cell and nuclei finder tools to detect and quantify in parallel the total number of CellMask-stained RBCs forming the monolayer and the Syto-stained, Mitotracker-positive gametocytes. Use morphology and intensity parameters for precise selection of RBCs and viable gametocytes. Viable gametocytes are defined as elongated, CellMask-positive objects with Syto 40 Blue and Mitotracker Deep Red FM signals with intensity values ≥350 and ≥3,000, respectively.

Use mathematical formula 1 to calculate the gametocytocidal activity of pharmacological compounds from upstream imaging plates:

\[
100 \times \left[ \frac{(\text{Gametocyte % in compound well})}{(\text{Gametocyte % in control wells})} \right]
\]

(1)

Killing rates >50 and >65% are routinely measured when mature gametocytes are exposed for 2 and 24 h, respectively, to 50 µM Gentian Violet. Use the mathematical formula 2 to calculate retention rates based on the percentage of gametocytes detected in upstream and downstream imaging plates:

\[
100 \times \left( \frac{(\text{Gametocyte %, Up well})}{(\text{Gametocyte %, Ds well})} \right)
\]

(2)

Averaged retention rates routinely fluctuate between 35 and 55%, 60 and 80%, and 90 and 95% after exposure to 0.05% (vol/vol) DMSO, 50 nM calyculin A and 5 µM NITD609, respectively. Exclusion criteria for the analysis of results from one compound plate are defined as a difference of <15% between the average retention rates of gametocytes from the DMSO and calyculin A control conditions; an average retention >60% for gametocytes within the DMSO control wells; an average retention <85% for gametocytes within the NITD609 control wells; an average killing rate <50 after exposure of mature gametocytes to 50 µM Gentian Violet.

**PROCEDURE**

**Automated loading of microspheres into frits-bottom filtration plates**

1| **TIMING 20 min per plate**

1| Place two empty dispenser reservoirs and the frits-bottom filtration plate coupled to a reservoir plate on the deck of the Biomek NX® dispenser. Place another dispenser reservoir on the dispenser’s deck and fill it with 100 ml of PBS-supplemented with 1% Albumax (PBS-A = suspension medium for microspheres).

2| Rotate on a wheel (40 rotations per min for at least 5 min) two Falcon tubes, each filled with 100 g of 25- to 45-µm microspheres in 25 ml of PBS-A. Keep the rotation going until microsphere use.

3| Repeat Step 2 with 5- to 15-µm microspheres (two tubes each containing 100 g of microspheres in 25 ml of PBS-A). Keep the rotation going until microsphere use.

4| Prefill the frits-bottom filtration plate from Step 1 with 60 µl of PBS-A, using the transfer function of the automated dispenser (Table 1).

▲ **CRITICAL STEP** Check transfer success visually. If necessary, gently tap the side of the microplate by hand to ensure homogeneous distribution of PBS-A over the entire frits surface.

? **TROUBLESHOOTING**

5| Check visually for complete resuspension of microspheres in the two Falcon tubes from Step 2 (a homogeneous gray liquid suspension should be observed), then transfer the resuspended 25- to 45-µm microspheres into one of the two empty dispenser reservoirs from Step 1. Immediately transfer this suspension of microspheres from the dispenser reservoir to the frits-bottom filtration plate from Step 1, using the aspirate–dispense function of the automatic dispenser (Table 1). When the microspheres appear to be fully sedimented in the wells (=10 s), tap the side of the microplate by hand very gently to ensure that the microsphere layer homogeneously covers the surface of the frits in all wells.

▲ **CRITICAL STEP** To ensure process reproducibility, automated microsphere loading into the microplate should be run immediately after microsphere transfer to the dispenser reservoir.

? **TROUBLESHOOTING**
6] Transfer the resuspended 5- to 15-µm microspheres in the two Falcon tubes from Step 3 to the other empty dispenser reservoir from Step 1 and immediately proceed to their automated transfer from the dispenser reservoir to the same frits-bottom filtration plate from Step 5 (adding them on top of the existing layer of microspheres), by repeating Step 5 (Table 1). The frits-bottom filtration plate loaded with both 25- to 45-µm and 5- to 15-µm microspheres is now termed the microsphiltration plate.

**TROUBLESHOOTING**

7] Use a hair dryer to remove surface bubbles, then the Beckman vacuum-filtration device to flow through most of the PBS-A from the microsphiltration plate from Step 6 to the reservoir plate. Put the bottom of the microsphiltration plate from Step 6 in close contact with absorbing paper for 5 min to achieve removal of excess medium by capillarity.

▲ **CRITICAL STEP** Inspect the layers of microspheres visually and identify ‘deficient’ wells that may display microsphere leak across the frits. Prepared microsphiltration plates routinely display <4 deficient wells (≤1% of the total plate wells). Seal deficient wells by melting their director nozzles with a soldering iron (Supplementary Fig. 5).

**TROUBLESHOOTING**

### Storage of microsphiltration plates

8] Process the microsphiltration plates differently for extemporaneous (option A) or deferred use (option B).

**(A)** Short-term storage for extemporaneous use ● **TIMING** 5 min per plate

(i) Store the plate at 4 °C in a sealed plastic bag with absorbing paper for up to 48 h.

**(B)** Mid- to long-term storage ● **TIMING** overnight + 10 min per plate

(i) Allow the microsphere-loaded plate to dry overnight on a slide warmer set at 60 °C in a class II microbiological safety cabinet (15–18 h).

▲ **CRITICAL STEP** Ventilation is critical to enabling complete drying of the microsphere layers.

**TROUBLESHOOTING**

(ii) Pack the microsphiltration plate in a moisture barrier bag containing a 50-g silica bead sachet. Seal the bag using a warm sealer.

(iii) Store at room temperature.

▲ **CRITICAL STEP** Avoid unnecessarily banging or disturbing the plates during transport and experiments.

■ **PAUSE POINT** Microsphiltration plates can be stored at room temperature for up to 6 months.

### Isolation of mature gametocytes by density-gradient centrifugation ● **TIMING** 1 h

▲ **CRITICAL** Sterility of the gametocyte culture should be maintained by working in a class II microbiological safety cabinet and using sterile reagents.

▲ **CRITICAL** Using a slide warmer, a block heater and reagents at 37.5 °C is necessary to prevent temperature drop, which is detrimental to mature gametocytes.

▲ **CRITICAL** In this section of the protocol, reagent volumes needed to process one gametocyte culture flask are given. Three gametocyte culture flasks are routinely prepared for an assay run of five 384-microsphiltration plates.

9] Remove ~75% of the gametocyte culture medium from the culture flask and use a 50- to 200-µl drop to prepare a Giemsa-stained blood film for quantification of gametocytemia in Step 13. To prepare a Giemsa-stained blood film for quantification of gametocytemia in Step 13. To prepare a Giemsa-stained blood film, place a drop of blood onto the edge of a clean glass slide and use another slide to rapidly smear the blood drop. Dry and fix the smear using 99% (vol/vol) methanol, then dip it for 3–5 min in a staining jar filled with Giemsa’s Azur Eosin Methylene Blue solution diluted five times with dH2O. Wash the smear with water, dry it using a hair dryer and store it in a referenced slide box at room temperature.

■ **PAUSE POINT** The smear can be stored up to two years when the humidity levels are ≤75%.

10] Gently swirl the mature gametocyte culture flask by hand over a slide warmer set at 37 °C to resuspend its contents. Transfer the gametocyte culture to a 50-ml Falcon tube prefilled with 30 ml of fresh and prewarmed gametocyte culture medium and centrifuge at 1,000 g, 5 min, 37 °C.

11] Discard the supernatant and gently resuspend the 1.5- to 2-ml pellet with fresh and prewarmed gametocyte culture medium to reach a 9-ml final volume.

12] Carefully and slowly, transfer a 4.5-ml volume of the washed gametocyte culture suspension to each of two 15-ml Falcon tubes that contain 10 ml of prewarmed Nycoprep solution. Do not mix; centrifuge at 200g, 20 min, 37 °C.

▲ **CRITICAL STEP** The tubes must be handled carefully and should not be disturbed in order to preserve a clear separation between the Nycoprep solution and the gametocyte culture.
During the centrifugation, proceed to quantification of gametocytemia from your Giemsa-stained film (prepared during Step 9). For this purpose, use the ×20–×40 magnification of an inverted bright-field microscope to record the total number of gametocyte-infected RBCs and the total number of RBCs counted in ten or more fields of the Giemsa-stained film. Calculate the gametocytemia level (percentage of gametocyte-infected RBCs) by dividing the total number of gametocytes by the number of RBCs and multiplying the calculated product by 100.

**CRITICAL STEP** Counting against >5,000 RBCs using quality stained films is recommended for precise quantification of gametocyte percentage.

Using a Pipetman at slow aspiration speed, transfer and combine the gametocyte phases from the two tubes from Step 12 (Fig. 5) in a 50-ml Falcon tube prefilled with 25 ml of prewarmed gametocyte culture medium.

**CRITICAL STEP** Using a Pipetman at slow aspiration speed is recommended to prevent dispersion of the gametocyte phase upon pipetting.

**CRITICAL STEP** A maximum 3- to 4-ml excess of culture medium around the gametocyte phase is recommended for complete retrieval of isolated gametocytes.

### Dilution of isolated gametocytes in fresh, uninfected RBCs

**TIMING 1 h**

15| Dilute isolated gametocytes with fresh uninfected RBCs by adding 30 µl of packed and fresh uninfected RBCs if gametocytemia is in the 1–3% range or 50 µl of packed and fresh uninfected RBC if gametocytemia is in the 3–6% range.

16| Wash the population of isolated prediluted gametocytes twice by centrifugation at 1,000 g, 5 min, 37 °C, using 2 volumes of fresh and prewarmed gametocyte culture medium.

17| Estimate the volume of the isolated prediluted gametocyte pellet using a manual micropipette.

18| Prepare a Giemsa-stained blood film and quantify gametocytemia, as described in Steps 9 and 13.

19| Add an appropriate volume of packed and fresh uninfected RBCs to reach a 3–5% final gametocytemia level, then prepare a last Giemsa-stained blood film to further control dilution accuracy (as described in Steps 9 and 13).

**CRITICAL STEP** To ensure dilution accuracy, prediluting, recounting and diluting again is recommended here when >15% of gametocytes are quantified by Giemsa-stained film (from Step 18).

### Troubleshooting

20| Finely adjust the hematocrit to 0.5% using a hemocytometer and transfer the gametocyte suspension to a culture flask for overnight maintenance in an incubator (37 °C, 5% O₂, 5% CO₂).

### Pharmacological exposure

**TIMING 3–4 h or 25–26 h, 1–2 h hands-on**

**CRITICAL** For a 2-h exposure with compounds, loading one 384-compound plate with gametocytes every 20 min for up to five plates is recommended to respect timing and microsphiltration run limits. For exposures lasting >24 h, five to ten compound plates can be routinely loaded at a time.

21| Remove from the freezer the stored polypropylene plates containing test compounds and in-plate controls ≥45-fold concentrated in 100% DMSO, as compared with final assay concentrations. Leave these at room temperature for 20 min on a bench.

22| Transfer the gametocyte suspension from Step 20 to a prewarmed 50-ml Falcon tube maintained at 37 °C on a block heater.

23| Transfer the gametocyte suspension (20 ml is required to fill one compound plate) from the Falcon tube to a plastic reservoir tray on a slide warmer set at 37 °C. Immediately remove the adhesive sealing film from the compound plate and
dispense 45 μl of gametocyte suspension into the wells, using an electronic multichannel pipettor with the multidispense function.  
**CRITICAL STEP** After transfer to reservoir tray, avoid gametocyte sedimentation by proceeding immediately to dispensing into compound plates.

24] Cover the compound–gametocyte plate using a sterile plastic lid or a Breathe-Easy sealing membrane and place the plate back into the incubator for 2 or 24 h.

**Preparation of imaging plates**  
**TIMING 30 min**  
25] The morning of the microsphiltration, transfer 12 μl of upstream staining solution and 16 μl of downstream staining solution (Reagent Setup) to the wells of 5 upstream and 5 downstream imaging plates, respectively. Store at room temperature protected from light until use by that afternoon.  
**CRITICAL STEP** Preparation of five upstream plus five downstream imaging plates is required for the microsphiltration of gametocyte-infected RBC samples from five compound plates.  
**CRITICAL STEP** Imaging plates are loaded with staining solutions that were prepared in advance and stored for no more than 24 h (as described in Reagent Setup).

**Rehumidification of microsphiltration plates**  
**TIMING 2–3 min**  
26] Place a reservoir filled with prewarmed gametocyte culture medium and a microsphiltration plate from Step 8 coupled to a reservoir plate on the Biomek dispenser workstation deck.

27] Using the transfer function of the automated dispenser, transfer 50 μl of prewarmed culture medium to the microsphiltration plate wells (Table 2).

28] Use the Beckman vacuum-filtration device to flow through the content of the microsphiltration plate into the reservoir plate.

29] Using the transfer function of the automated dispenser, replace the first reservoir plate with a new sterile one prefilled with 25 μl of culture medium per well (Table 2).

**Resuspension and transfer of gametocytes samples to microsphiltration plates**  
**TIMING 2–3 min**  
30] Place the gametocyte-compound plate from Step 24 as well as upstream and downstream imaging plates from Step 25 on the deck of the Biomek NXP® dispenser (Fig. 6).

31] Using the transfer function of the automated dispenser, add 25 μl of culture medium from Step 26 to the top of the microsphere layers of the microsphiltration plate (Table 2).

32] Using the aspirate–dispense function of the automated dispenser, resuspend the sedimented gametocyte culture samples from the gametocyte-compound plate (Table 2).  
**CRITICAL STEP** To obtain reproducible resuspensions with >80% efficiency, it is recommended to use 5x aspirate–dispense cycles targeting each pole and the center of the cell layer surface, with a distance of 0.5–1.5 mm between the cell layers and the dispenser tip ends (Table 2).  
**TROUBLESHOOTING**

33] Transfer 25 μl of the resuspended gametocyte samples from the gametocyte-compound plate into the microsphiltration plate using the transfer function of the dispenser (Table 2). Thereafter, run tip wash sequence.

**Dilution of upstream unfiltered control samples (upstream samples) and transfer to upstream imaging plates**  
**TIMING 5 min**  
34] Immediately transfer 80 μl of culture medium to the wells of the gametocyte-compound plate and mix the diluted suspensions, using the transfer and mix function of the automated dispenser (Table 2).
35| Using the transfer and mix function of the automated dispenser, transfer 12 µl of the diluted upstream samples to the upstream imaging plate (Table 2). Thereafter, run the tip wash sequence.

**Filtration across microsphere layers and rinsing step ● TIMING 5 min**
36| Use the Beckman vacuum-filtration device to filter resuspended gametocyte samples in the microsphiltration plate from Step 33.

37| Gently tap the reservoir plate against the Biomek deck to enable the drops of sample, which are potentially on the well sides, to slide over the reservoir plate bottom.

38| Using the automated dispenser, transfer 80 µl of culture medium from Step 26 to the wells of the microsphiltration plate (Table 2) and use the Beckman vacuum-filtration device to flow through the culture medium into the reservoir plate from Step 37 (rinsing step; Fig. 1d).

**Transfer of downstream filtered samples (downstream samples) to downstream imaging plates ● TIMING 2–3 min**
39| Using the transfer and mix function of the automated dispenser, remove the microsphiltration plate from the top of the reservoir plate containing the downstream samples. Transfer 16 µl of downstream samples from the reservoir plate to the downstream imaging plate (Table 2). Thereafter, run tip wash sequence.

40| Incubate both upstream and downstream imaging plates at 24–28 °C overnight, protected from light with an aluminum film.

**High-throughput imaging ● TIMING 90 min per imaging plate**
41| Set up the Opera high-resolution spinning disc confocal imager as described in the Equipment Setup section. Select the method and the exposure and measurement parameters described in Table 3.

42| Run imaging of control wells from upstream and downstream imaging plates using the automated imaging protocol discussed in the Equipment Setup section, which provides gametocytemia levels from the imaged fields.

43| Check calculated retention rates and killing activities from the positive and negative control wells to establish whether these match assay quality-control criteria defined in the Equipment Setup section.

**TROUBLESHOOTING**
44| Once in-plate controls have demonstrated appropriate reference values, load upstream and downstream imaging plates on the automated stacker with barcode reader and start walk-away imaging of test samples.

**TROUBLESHOOTING**
Troubleshooting advice can be found in Table 4.

**TABLE 4 | Troubleshooting table.**

<table>
<thead>
<tr>
<th>Step</th>
<th>Problem</th>
<th>Possible reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Heterogeneous volumes of PBS-A transferred to filter-bottom plates</td>
<td>Tips are not completely filled with PBS-A</td>
<td>Add a delay time after PBS-A aspiration to fill all tips evenly</td>
</tr>
<tr>
<td>5</td>
<td>Heterogeneous volumes of 25- to 45-µm spheres transferred to filter-bottom plates</td>
<td>Tips are loaded with homogeneous volumes of spheres but do not empty evenly</td>
<td>Increase move speed of the dispenser pipetting head and/or increase dispensing speed</td>
</tr>
<tr>
<td>6</td>
<td>Heterogeneous volumes of 5- to 15-µm spheres transferred to filter-bottom plates</td>
<td>Heterogeneous sphere volumes in tips</td>
<td>Add a delay time after aspiration and slow the aspiration speed</td>
</tr>
<tr>
<td>7</td>
<td>Excess of ‘deficient wells’ observed consistently</td>
<td>25- to 45-µm sphere layer is insufficiently thick</td>
<td>Increase the volume of 24- to 45-µm spheres transferred to the filter-bottom plate (Step 1A(iv))</td>
</tr>
</tbody>
</table>

(continued)
**TABLE 4 | Troubleshooting table (continued).**

<table>
<thead>
<tr>
<th>Step</th>
<th>Problem</th>
<th>Possible reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>8B(i)</td>
<td>Sphere layers appear yellow–green colored after drying</td>
<td>Sphere layers oxidized during drying and the microsphiltration plate should be discarded</td>
<td>Reduce the number of microsphiltration plates that are dried over one slide warmer. Increase airflow speed of the ventilated compartment</td>
</tr>
<tr>
<td>19</td>
<td>Presence of morphologically viable <em>P. falciparum</em> asexual stages after Nycoprep</td>
<td>Starting gametocyte percentage &lt;1% Excessive retrieval of media surrounding the gametocyte phase</td>
<td>Discard the culture and refer to Delves’ protocol if the problem persists. Reduce medium retrieval to the gametocyte phase only</td>
</tr>
<tr>
<td></td>
<td>Gametocyte level is &lt;3% after dilution in fresh, uninfected RBCs</td>
<td>Overdilution induced by overestimation of gametocyte percentage after Nycoprep isolation</td>
<td>Prepare multiple Giemsa-stained films and increase the total number of RBCs considered for quantification</td>
</tr>
<tr>
<td></td>
<td>Gametocytes appear rounded</td>
<td>Gametocytes were activated during Nycoprep isolation and/or dilution in RBCs</td>
<td>Prewarm the centrifuge, material and reagents to 37 °C before use with mature gametocytes to prevent detrimental alterations induced by temperature drop</td>
</tr>
<tr>
<td>32</td>
<td>Low yield of RBC layer resuspension</td>
<td>Suboptimal position of tips above the RBC layer</td>
<td>Reduce distance between the dispenser tips and the RBC layer. Increase mixing speed</td>
</tr>
<tr>
<td>43</td>
<td>Low cell density in imaging plate</td>
<td>Suboptimal RBC resuspension prior to microsphiltration Insufficient number of RBCs transferred to imaging plate</td>
<td>Improve the automated sequence for resuspension (i.e., Step 4B(iii)). Increase the volume of compound-exposed gametocytes transferred to the imaging plate</td>
</tr>
</tbody>
</table>

**TIMING**
Steps 1–7, plate preparation: 20 min per plate
Step 8A, short-term storage: 5 min per plate
Step 8B, long-term: overnight + 10 min per plate
Steps 9–20, isolation and dilution of gametocytes: 2 h
Steps 21–24, pharmacological exposure: 1–2 h hands-on + 2 h or 24 h of exposure
Step 25, preparation of imaging plates: ~30 min
Steps 26–40, microsphiltration: ~20 min per gametocyte-compound plate
Steps 41–44, imaging and analysis: 90 min per plate

**ANTICIPATED RESULTS**
In the protocol described here, gametocytes are produced as according to the protocol of Delves et al. in which medium culture flasks are initially seeded (day 0) with 2 ml of packed RBCs parasitized by 0.5% *P. falciparum* ring stages at 4% hematocrit, then maintained over 2 weeks, with daily culture medium change. The gametocyte induction protocol is routinely started on a Thursday or Friday to proceed to Nycoprep isolation of gametocytes 17 or 18 d later (on a Monday). With this method, ~1.5 ml of packed RBCs infected with mature stage V gametocytes are harvested from each culture flask at day 17 or 18, from which gametocytes are prepared as above.

Figure 7 | Expected results from the microsphiltration assay. (a) Scatter plot illustrating normalized screen data from one microsphiltration plate. Mature gametocytes were exposed to 1 µM pharmacological compounds (simplicate) during 2-h prior filtration across a 384-well microsphiltration plate, using 0.05% (vol/vol) DMSO (blue squares), 50-nM calyculin A (green triangles), 5-µM cipargamin (NITD609, KAE609; orange diamonds) and 50 µM Gentian Violet (purple diamonds) as controls. (i) Hit selection window in which hits were defined as compounds increasing gametocyte retention rates (RRs) to >62.06% (DMSO mean + 2 s.d.) with a killing rate inferior to 20.12% (DMSO mean + 4 s.d.). (ii) Z-factor values from one microsphiltration assay run assessing 2- or 24-h activities of 1,056 compounds (3× microsphiltration plates) tested at 1 µM on mature gametocytes.
which three 384-microsphiltration plates can commonly be run. Experimenters can produce up to ten microsphiltration plates per day using the automated method. Stored microsphiltration plates show optimal filtration performance over at least 6 months after their loading with microsphere layers. Microsphiltration plate shipment has recently been demonstrated to be feasible internationally for field malaria studies. Using a previously published protocol for continuous gametocyte production in vitro, this assay enables screening of up to 3,600 compounds per week using ten 384-well microsphiltration plates. Because this screening methodology applies to an essentially quiescent parasite population, a low hit rate (<0.1–1%) is expected. The Z factor is predicted to fluctuate between 0.4 and 0.7 (Fig. 7). Microsphiltration offers the possibility of adapting the experiment readout to any RBC population of interest. When using a flow cytometer equipped with a robotic arm to quantify retention rates of RBC samples (Supplementary Figs. 6–8), up to 1,152 data points can be generated in 1 working day.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS P.B. supervised the overall project as principal investigator and supported the design and analysis of experiments. J.D., M. Carucci and I.G.-B. designed and analyzed the experiments under the supervision of P.B., F.-J.G., L.S., N.B.R. and P.A.N. J.D. wrote the paper with support provided by P.B. M. Carucci and I.G.-B. supported the preparation of figures and tables. C.R. and B.H. performed the microsphiltration of RBC samples from human donors and prepared Supplementary Figure 6. M. Corral and O.P. ensured automation maintenance, provided technical support and machined the platform used to confine the Biomek dispenser. J.L.P. provided expertise for data analysis.

COMPETING INTERESTS The authors declare no competing interests.

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