Ring-stage Survival Assays (RSA) to evaluate the *in-vitro* and *ex-vivo* susceptibility of *Plasmodium falciparum* to artemisinins

Procedure

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Version History

Version number	Revision(s) & reason for amendment	Release date
1.0	Creation of procedure	11 September 2013
1.1	Expansion of data collection methods to include flow cytometry	29 April 2014
1.2	Addition of final concentration measures for DMSO and DHA solutions. Section 6.5.2	27 October 2014

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Contents

1. Purpose

This procedure describes two assays to evaluate the susceptibility of *Plasmodium falciparum* to artemisinins. The ***in-vitro* Ring-stage Survival Assay (RSA$^{0\text{-}3h}$)** is performed on 0-3 hour post-invasion rings obtained from culture-adapted parasites. The ***ex-vivo* Ring-stage Survival Assay** (***ex-vivo* RSA**) is performed on parasite isolates freshly collected from patients with malaria. In both assays, parasites are exposed to 700 nM dihydroartemisinin (DHA) for 6 hours – approximating their drug exposure in patients treated with an artemisinin – and their survival is assessed 72 hours later as described in Witkowski, Amaratunga *et al.* 2013 (1), with minor modifications.

2. Scope

This procedure details the steps required to determine the percentage of viable parasites at 72 hours, following a 700-nM, 6-hour pulse of DHA. Samples suitable for testing are either adapted parasite lines (synchronized at the 0-3 hour post-invasion ring stage) in the *in-vitro* RSA$^{0\text{-}3h}$ or clinical parasite isolates (freshly obtained from patients with malaria and *P. falciparum* monoinfection) in the *ex-vivo* RSA.

3. Abbreviations

ACD	acid citrate dextrose
DHA	dihydroartemisinin
DMSO	dimethyl sulfoxide
FBS	fetal bovine serum
HBSS	Hank's balanced salt solution
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
MT	MitoTracker® Deep Red FM
PBS	phosphate-buffered saline
QA	quality assurance
QC	quality control
RBC	red blood cell
RPMI	Roswell Park Memorial Institute
RSA	ring-stage survival assay
RT	room temperature
SG	SYBR® Green I
WBC	white blood cell
BP	Band-pass
L	liter
mL	milliliter
µL	microliter
µg	microgram
ng	nanogram
mM	millimolar
µM	micromolar
nM	nanomolar
µm	micrometer
nm	nanometer

MW	molecular weight
U	unit

4. Duties and Responsibilities

This procedure is designed for use by appropriately-equipped laboratories that perform *in-vitro* and *ex-vivo* drug susceptibility tests on *P. falciparum*. To perform the procedure successfully, training is required and participation in an appropriate QA/QC proficiency scheme is recommended.

5. Materials and Equipment

5.1. Materials

General

- Sterile pipettes (2 mL, 5 mL, 10 mL, 25 mL)
- Sterile tips (10 µL, 20 µL, 200 µL, 1000 µL)
- Sterile transfer pipettes
- Cell culture flasks with vented cap (25 cm^2)
- Cell culture flasks with vented cap (75 cm^2)
- 48-well culture plates
- Microscope slides
- Microscope slide staining jars
- Centrifuge tubes (15 mL, 50 mL)
- Microcentrifuge tubes (1.5 mL)
- Polystyrene round-bottom tubes (5 mL)
- Disposable sterilization filter units, 0.22 µm, 1 L capacity
- 70% ethanol

Blood sample collection

- ACD Vacutainers® (Becton-Dickinson, #364816)
- BD Vacutainer® Safety-Lok™ Blood Collection Set (Becton-Dickinson, #364815)
- BD Vacutainer® One Use Holder (Becton-Dickinson, #367281)

Parasite culture

- RPMI-1640 + GlutaMAX™-1 (Gibco, #61870-044) stored at 4°C
- Albumax II (Invitrogen, #11021-037) stored at 4°C
- Sterile human serum (ABO/Rh blood group 0+) stored at –20°C
- RBCs (ABO/Rh blood group 0+) stored at 4°C
- HEPES Buffer solution 1 M (Gibco, #1563-056, 100 mL) stored at 4°C
- Gentamicin solution, 10 mg/mL (Invitrogen, #15710-072) stored at RT
- Hypoxanthine 10 mM (c·c·pro, #Z-41-M, 100 mL) stored at 4°C
- Distilled water for cell culture (Gibco, #10977-035, 500 mL) stored at RT
- Methanol stored at RT
- Giemsa stain (Merck, #1.09204.0500, 500 mL) stored at RT
- Glycerolyte 57 (Baxter, #4A7833) stored at RT

Parasite synchronization

- 10X PBS (Gibco, #70013-016, 500 mL) stored at RT
- Heparin sodium 5,000 U/mL (Rotexmedica, #3862340) stored at 4°C
- Percoll (Sigma, #P4937) stored at 4°C
- D-sorbitol (Sigma, #S1876) stored at RT

Ring-stage Survival Assay (RSA)

- Dihydroartemisinin (provided by WWARN) stored at 4°C
- DMSO (Sigma, #D2650) stored at RT
- Methanol (Sigma, #32213, 1 L) stored at RT
- Rapid Giemsa stain (RAL diagnostics, #BLUE-RAL 555 and #EOSIN-RAL 555) stored at RT
- Giemsa stain (Merck, #1.09204.0500, 500 mL) stored at RT
- 10X HBSS (Gibco, #14065-056, 500 mL) stored at RT
- FBS (Sigma, #F2442, 100 mL) stored at -20°C
- 10,000X SYBR® Green I nucleic acid gel stain (Invitrogen, #S-7563, 500 µL) stored at -20°C
- MitoTracker® Deep Red FM (Molecular Probes, #M22426, 20 x 50 µg) stored at -20°C

5.2. Equipment

- Pipettor
- Micropipettes
- Water-jacketed incubator at 37°C with tri-gas mixture (5% CO_2, 5% O_2, 90% N_2)
- Class II biosafety cabinet
- Vortexer
- Centrifuge with swinging bucket rotor (e.g., Eppendorf 5702)
- Microcentrifuge
- Oil-immersion microscope
- Flow cytometer with 488-nm laser, 640-nm laser, 530 ± 15-nm BP filter, and 675 ± 12.5-nm BP filter (e.g., BD Accuri C6)
- Flow cytometry data analysis software (e.g., FlowJo vX, Tree Star Inc.)
- Freezer (–20°C or below)
- Refrigerator
- Water bath
- Weighing balance

6. Procedure

6.1. Aseptic procedures

- All procedures (except centrifugation, vortexing, 37°C incubations, and preparing stained blood smears) are performed in a Class II biosafety cabinet, which is wiped down with aseptic solution at the start and end of each day.
- The incubator and storage surfaces are cleaned with aseptic solution at least every 3 months.

6.2. Reagent preparation

6.2.1. Preparation of culture medium

- Prepare the culture medium as described in Table 1.
- Use a magnetic stirrer to ensure that components are completely dissolved.
- Heat-inactivate the human serum at 56°C for 30 minutes prior to use.
- After preparation, filter the medium using a 0.22 µm pore size, 1 L filter.
- Store at 4°C for up to 2 weeks.

Table 1. *Volumes of reagents for 1 L of culture medium*

Reagent	Stock solution	Volume/weight
RPMI-1640 medium		928 mL
HEPES	1 M	25 mL
Gentamicin	10 mg/mL	2 mL
Hypoxanthine	10 mM	20 mL
Albumax II		5 g
Heat-inactivated human serum		25 mL

6.2.2. Preparation of DHA solution

1. Resuspend the DHA (provided by WWARN as a pre-weighed ~1 mg aliquot) in DMSO to obtain a 1 mg/mL stock solution. Vortex to dissolve completely.
2. Dilute the DHA stock solution 5-fold in DMSO to obtain a 200 µg/mL solution. Vortex.
3. Make 50 µL aliquots in sterile 1.5 mL microcentrifuge tubes.
4. Store at –20°C for up to 6 months.

6.2.3. Preparation of uninfected red blood cells

- RBCs are used to lower parasitemia and adjust hematocrit as needed in parasite cultures and RSAs.
- RBCs should test negative for hemoglobinopathies and G6PD deficiency before use.
- Collect whole blood in a 250 mL pouch containing ACD anticoagulant.
    1. Spray the blood pouch with 70% ethanol and let it dry in the biosafety cabinet.
    2. Open carefully by cutting the pouch's tube with sterile scissors.
    3. Transfer the blood into 50 mL centrifuge tubes.
    4. Store at 4°C for up to 3 weeks.
- Before using RBCs in parasite cultures and RSAs:
    1. Rule out hemoglobinopathies and G6PD deficiency.

2. Centrifuge 25 mL of whole blood at 1000 g for 10 minutes.
3. Remove the plasma and buffy coat.
4. Add cold (4°C) RPMI to a final volume of 50 mL.
5. Centrifuge at 1000 g for 10 minutes.
6. Remove the supernatant.
7. Repeat steps 4-6 twice.
8. Add 3 mL of RPMI to RBC pellet.
9. Centrifuge at 1000 g for 10 minutes.
10. Store (without mixing the cells) at 4°C up to 4 days.

6.2.4. Preparation of 5% sorbitol solution

1. Add 50 g of D-sorbitol to 1 L of culture water.
2. Stir until completely dissolved.
3. Sterilize the solution using a 0.22 µM pore size, 1 L filter.
4. Store at 4°C up to 1 year.

6.2.5. Preparation of Percoll® solution

These steps are done at RT, in a Class II biosafety cabinet, and using sterile reagents.

1. Mix 9 volumes of Percoll with 1 volume of 10X PBS to make Percoll 90% solution. Mix thoroughly. Store at 4°C up to 2 months.
2. Freshly prepare heparinized RPMI by adding 15 µL heparin (5,000 U/mL) to 10 mL RPMI.
3. Freshly prepare Percoll 75% solution by adding 1.5 volumes of heparinized RPMI to 7.5 volumes of Percoll 90% solution.

6.2.6. Preparation of Giemsa stain

- Giemsa (Merck) – Add 1 volume of Giemsa to 9 volumes of 0.4% NaCl solution. Stain smears for 20 minutes and rinse.

6.2.7. Preparation of flow cytometry sample wash solution

These steps are done at RT, in a Class II biosafety cabinet, and using sterile reagents.

1. Add 5 mL of 10X HBSS to 45 mL of culture water to make a 1X HBSS solution.
2. Add 1 mL of FBS to 49 mL of 1X HBSS.
3. Store at 4°C for up to 2 weeks.

6.2.8. Preparation of SG solution

1. Add 2.5 µL of 10,000X SG to 497.5 µL of 1X HBSS solution to make a 50X SG solution.
2. Store at -20°C for up to 6 months.
3. Add 100 µL of 50X SG solution to 900 µL of 1X HBSS solution to make a 5X SG solution.
4. Make 20 µL aliquots in 1.5 mL microcentrifuge tubes.
5. Store at -20°C for up to 6 months.

6.2.9. Preparation of MT solution

1. Add 92 µL of DMSO to a tube of MT (supplied as a 50 µg lyophilized powder) to make a 1 mM MT solution.
2. Store at -20°C for up to 6 months.
3. Add 6 µL of 1 mM MT solution to 794 µL of 1X HBSS solution to make a 7.5 µM MT

solution.

4. Make 20 µL aliquots in 1.5 mL microcentrifuge tubes.
5. Store at -20°C for up to 6 months.

***Table 2**. Information regarding the storage and shelf-life of reagents*

Reagent	Storage temperature	Shelf-life
Culture medium	4°C	2 weeks
Sorbitol 5% solution	4°C	1 year
DHA solution	–20°C	6 months
Percoll® 90% solution	4°C	2 months
Heparinized RPMI	RT	Freshly made
Percoll® 75% solution	RT	Freshly made
Uninfected RBCs in ACD	4°C	3 weeks
Uninfected RBCs for culture	4°C	4 days
Giemsa solution	RT	Freshly made
Flow cytometry sample wash solution	4°C	2 weeks
SG solution	–20°C	6 months
MT solution	–20°C	6 months

6.3. Collection and preparation of blood from malaria patients

All steps are performed at RT unless otherwise stated. All procedures following blood draw should be performed in a biosafety cabinet to avoid bacterial contamination.

6.3.1. Sample collection

- Aseptically draw venous blood into an ACD Vacutainer® using the BD Vacutainer® Safety-Lok™ Blood Collection Set and holder.
- Mix by gently inverting tube 5 times and store the blood at 4°C up to 24 hours.

6.3.2. Sample preparation

1. Prepare a thin blood smear using a drop of blood. Stain with Giemsa (section 6.2.6).
2. Evaluate the parasitemia.
3. Centrifuge the blood tube at 800 g for 5 minutes.
4. Remove the plasma and the buffy coat.
5. Transfer the RBC pellet to a 50 mL centrifuge tube.
6. Add 20 mL of RPMI (pre-warmed to RT).
7. Centrifuge at 800 g for 5 minutes.
8. Remove the supernatant.
9. Repeat steps 6-8 twice.

The infected RBCs are now ready to be processed for *ex-vivo* RSA, long-term storage (section 6.3.3), or culture-adaptation (section 6.4).

6.3.3. Preparation of P. falciparum samples for long-term storage

This step is optional but provides backup aliquots that can be used years later.

1. In a 50 mL centrifuge tube, add Glycerolyte (3X volume of RBC pellet) drop-wise at RT to the RBC pellet obtained in section 6.3.2.

2. Mix gently with a pipette.
3. Let it stand for 1 minute at RT.
4. Distribute 500 µL of the preparation into sterile cryotubes.
5. Freeze at –80°C for at least 18 hours.
6. Transfer vials to liquid nitrogen for long-term storage.

6.4. *In-vitro* culture adaptation

All steps are performed at RT unless otherwise stated. All procedures are performed in a level II biosafety cabinet to avoid bacterial contamination. **All *P. falciparum* samples are suitable for *in-vitro* culture adaptation, regardless of parasitemia.**

6.4.1. Freshly-collected samples

1. Warm culture medium to 37°C in a water bath.
2. Add 5 mL of culture medium to a 25 cm^2 culture flask.
3. Add 150 µL of the infected RBC pellet (section 6.3.2) and 50 µL of uninfected RBCs (section 6.2.3) to achieve a 4% hematocrit.
4. Mix gently.
5. Maintain the culture in an incubator under the following conditions: 37°C, humid atmosphere, 5% O_2 and 5% CO_2, 90% N_2.
6. Replace 5 mL of culture medium (pre-warmed to 37°C) daily. Check the parasitemia daily by making Giemsa-stained thin blood smears.
    a. If no or few parasites are detected (< 0.1% of the initial parasitemia), add 50 µL of uninfected RBCs (section 6.2.3) weekly in addition to replacing medium daily. Stop the culture if no parasites are observed in 60 days.
    b. If parasites are detected and are growing normally (parasitemia increases 1.5- to 10-fold every 48 hours), maintain the parasitemia under 3%. For assays, dilute the pellet with uninfected RBCs to achieve a 0.5-1% parasitemia and add culture medium to obtain a 4% hematocrit.
7. Adaptation is considered successful after 2 weeks of uninterrupted parasite culture.

6.4.2. Cryopreserved samples

1. Remove a tube containing cryopreserved *P. falciparum* parasites (section 6.3.3) from liquid nitrogen.
2. Thaw the vial in a 37°C water bath. Wear eye-protection. Avoid contact between water and the cap of the tube.
3. After thawing, transfer the solution to a 15 mL centrifuge tube.
4. Centrifuge at 800 g for 5 minutes.
5. Remove the supernatant.
6. Add 1 volume (equal to the initial volume in the cryotube) of 3.5% NaCl solution drop-wise while shaking the tube gently.
7. Let it stand for 1 minute at RT.
8. Add 12 mL of RPMI (pre-warmed to 37°C) and mix gently.
9. Centrifuge at 800 g for 5 minutes.
10. Remove the supernatant.
11. Resuspend the RBCs in 10 mL of culture medium (pre-warmed to 37°C).
12. Transfer to a 25 cm^2 culture flask and add uninfected RBCs to obtain a 2% hematocrit.
13. Maintain the culture in an incubator under these conditions: 37°C, humid

atmosphere, 5% O_2, 5% CO_2 and 90% N_2, and follow the procedure in section 6.4.1.

6.5. *Ex-vivo* RSA

All steps are performed at RT unless otherwise stated. All procedures are performed in a sterile biosafety cabinet to avoid bacterial contamination. ***P. falciparum* samples with parasitemia ≥ 0.1% are suitable for this assay.**

6.5.1. Preparation of P. falciparum sample and drug solution

- Prepare a parasite solution by adding 50 µl of infected RBCs (section 6.3.2) to 2.5 mL of culture medium.
- If the parasitemia is > 1%, dilute the pellet with uninfected RBCs to obtain a 0.5-1% parasitemia and with culture medium to obtain a 2% hematocrit.
- Make a thin smear (= initial parasitemia, "INI").
- Prepare the DMSO control solution by adding 20 µL of DMSO to 2 mL of culture medium. Prepare the DHA test solution by adding 20 µL of DHA stock solution (section 6.2.2) to 2 mL of culture medium. Mix both solutions by vortexting.
- Do not re-use the thawed aliquot of DHA. Always use a fresh aliquot.

6.5.2. Ex-vivo RSA

1. In a 48-well culture plate, add:
   - 100 µL of DMSO solution in the ''non-exposed'' well (final concentration 0.1%) and
   - 100 µL of DHA solution in the ''DHA-exposed'' well (final concentration 700 nM).
2. Add 900 µL of infected RBC suspension to each well.
3. Mix gently with pipette while avoiding contact between wells.
4. Maintain the 48-well culture plate in an incubator under these conditions: 37°C, humid atmosphere, and 5% O_2, 5% CO_2 and 90% N_2 for exactly 6 hours.
5. After 6 hours, transfer the contents of the wells into 15 mL centrifuge tubes (non-exposed and DHA-exposed).
6. Centrifuge at 800 g for 2 minutes.
7. Remove the supernatant.
8. Add 12 mL of RPMI (pre-warmed at 37°C) and resuspend the RBC pellet.
9. Centrifuge at 800 g for 5 minutes.
10. Remove the supernatant.
11. Add 1 mL of culture medium (pre-warmed to 37°C) and resuspend the RBCs.
12. Transfer the suspension into 2 new wells in the 48-well culture plate.
13. Maintain the 48-well culture plate in an incubator under these conditions: 37°C, humid atmosphere, and 5% O_2 and 5% CO_2 and 90% N_2 for 66 hours.
14. Mix and transfer the culture from each well into a 1.5 mL microcentrifuge tube.
15. Centrifuge briefly (2000 rpm for 30 seconds).
16. Use 2 µL of the pellet and make a thin blood smear from both samples.
17. Fix the smears in methanol for 2 seconds and stain using Giemsa (section 6.2.6).

6.6. *In-vitro* RSA$^{0\text{-}3h}$

All steps are performed at RT unless otherwise stated. All procedures are performed in a level II biosafety cabinet to avoid bacterial contamination. **All *P. falciparum* samples are suitable for this assay, if the culture adaptation is successful.**

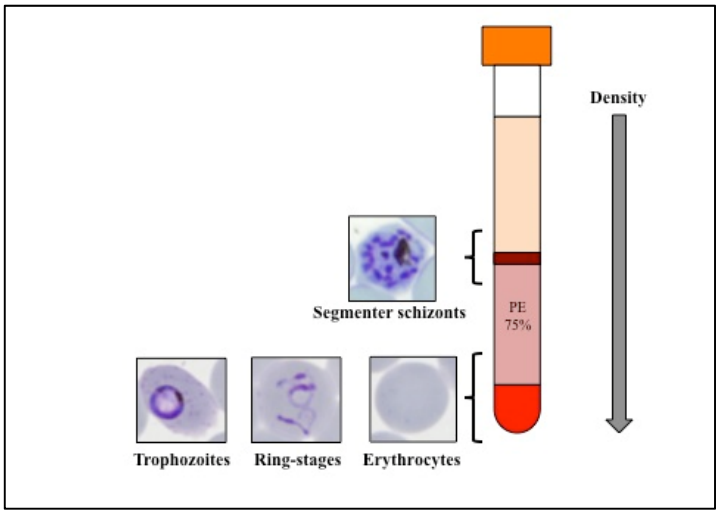
6.6.1. Preparation of 0-3 hour post-invasion rings

1. Use culture-adapted parasites (section 6.4) in a 25 cm^2 culture flask.
2. Allow the parasitemia to increase by changing the culture medium daily without adding uninfected RBCs.
3. Check the parasitemia daily; when it increases to 2-5%, transfer the culture to a 15 mL centrifuge tube.
4. Centrifuge at 800 g for 5 minutes.
5. Remove the supernatant.
6. Transfer the RBC pellet (~200 µL) to a 75 cm^2 culture flask containing 35 mL of culture medium (pre-warmed to 37^{o}C) and 500 µL of uninfected RBCs.
7. Increase the parasitemia up to 4% by changing the culture medium daily.
8. When the proportion of rings is up to 50%, transfer the culture to a 50 mL centrifuge tube.
9. Centrifuge at 800 g for 5 minutes.
10. Remove the supernatant.
11. Add 10 volumes of 5% sorbitol (pre-warmed to 37°C) to the RBC pellet (1 volume).
12. Incubate 10 minutes at 37°C and vortex for 5 seconds.
13. Centrifuge at 800 g for 5 minutes.
14. Remove the supernatant.
15. Transfer the RBC pellet to a 75 cm^2 culture flask containing 35 mL of culture medium (pre-warmed to 37°C).
16. Repeat steps 9-15, 30-48 hours later (depending on parasite development and stages at the time of sorbitol treatment).
17. After 30 hours of culture, make a thin blood smear and stain with Giemsa.
    - If the proportion of mature schizonts (10-12 nuclei) is > 0.5%, go to step 18.
    - If the proportion of mature schizonts (10-12 nuclei) is < 0.5%, repeat step 16.
18. Transfer the culture suspension to a 50 mL centrifuge tube.
19. Centrifuge the culture suspension at 800 g for 5 minutes.
20. Remove the supernatant.
21. Transfer 400 µL of the pellet to a 15 mL centrifuge tube and return the remaining pellet to culture conditions.
22. Add 4 mL of heparinised RPMI (pre-warmed to 37°C) (section 6.2.5) to the 400 µL pellet and mix gently.
23. Incubate for 15 minutes at 37°C.
24. In a 15 mL centrifuge tube, add 4 mL of 75% Percoll solution at RT.
25. Carefully layer the 4 mL RBC suspension in heparinised RPMI on top of the 75% Percoll solution (slant the 15 mL centrifuge tube at a very low angle and slowly add the RBC suspension drop-wise along the wall of the tube using a transfer pipette). If this step is correctly performed, you must observe two separated phases: the infected RBC suspension on the top, and the Percoll 75% solution on the bottom.
26. Centrifuge at 1000 g for 15 minutes. You must observe an intermediate phase containing the mature schizonts (**see Figure 1**).
27. Collect this phase carefully and transfer it to a 15 mL tube.
28. Add up to 13 mL of RPMI (pre-warmed to 37°C). Mix by inverting the tube.
29. Centrifuge at 800 g for 5 minutes.
30. Remove the supernatant.
31. Using 0.5 µL of the pellet, make a thin smear, fix it with methanol, and stain it with Giemsa (section 6.2.6). Schizonts must be concentrated (> 10%). Ring stages must

be rare (< 10%). While the slide is staining, proceed to steps 33-36.

32. Add 10 mL of culture medium (pre-warmed to 37°C) to the infected RBCs.
33. Add 200 µL of uninfected RBCs.
34. Transfer the RBC suspension to a 25 cm^2 flask.
35. Place in an incubator under these conditions: 37°C, humid atmosphere, 5% O_2, 5% CO_2 and 90% N_2 for <u>exactly</u> 3 hours.

Figure 1: *Differential density of parasites of different stages enabling segmenting schizont selection on 75% Percoll.*



<u>The following steps must be performed within 1 hour maximum.</u>

36. Mix the culture and transfer 0.5 mL into a 1.5 mL microcentrifuge tube.
37. Centrifuge 2000 rpm for 30 seconds.
38. Using 2 µL of the pellet make a thin smear, fix it with methanol, and stain it with Giemsa (Rapid stain RAL555 is preferred since it will take < 1 minute).
39. Quickly evaluate the proportion of ring-stages (must be > 0.5%).
40. Transfer the culture suspension to a 15 mL centrifuge tube.
41. Centrifuge at 800 g for 5 minutes. Remove the supernatant.
42. Add 10 volumes of 5% sorbitol (pre-warmed to 37°C) to the RBC pellet (1 volume).
43. Incubate for 10 minutes at 37°C and vortex for 5 seconds.
44. Centrifuge at 800 g for 5 minutes. Remove the supernatant.

6.6.2. Preparation of P. falciparum sample and drug solution

- If parasitemia from step 39 is > 1%, add uninfected RBCs and culture medium (pre-warmed to 37°C) to obtain a 0.5-1% parasitemia and 2% hematocrit.
- Make a thin smear (= initial parasitemia, "INI").
- Prepare the DMSO control solution by adding 20 µL of DMSO to 2 mL of culture medium. Prepare the DHA test solution by adding 20 µL of DHA stock solution (section 6.2.2) to 2 mL of culture medium. Mix both solutions by vortexting.
- Do not re-use the thawed aliquot of DHA. Always use a fresh aliquot.

6.6.3. In-vitro RSA^{0-3h}

1. In a 48-well culture plate, add:
    - 100 µL of DMSO solution in the ''non-exposed'' well and

- 100 µL of DHA solution in ''DHA-exposed'' well.

2. Add 900 µL of infected RBC suspension to each well.
3. Mix gently with a pipette while avoiding contact between wells.
4. Maintain the 48-well culture plate in an incubator under these conditions: 37°C, humid atmosphere, 5% O_2, 5% CO_2 and 90% N_2 for exactly 6 hours.
5. After 6 hours, transfer the contents of the wells to 15 mL centrifuge tubes (non-exposed and DHA-exposed).
6. Centrifuge at 800 g for 2 minutes.
7. Remove the supernatant.
8. Add 9 mL of RPMI (pre-warmed to 37°C). Resuspend the RBCs by inverting the tube.
9. Centrifuge at 800 g for 5 minutes.
10. Remove the supernatant.
11. Add 1 mL of culture medium (pre-warmed to 37°C) and mix to resuspend the RBCs.
12. Transfer the suspension into 2 new wells in the 48-well culture plate.
13. Maintain the 48-well culture plate in an incubator under the following conditions: 37°C, humid atmosphere, 5% O_2 and 5% CO_2 and 90% N_2 for 66 hours.
14. Mix and transfer the culture from each well into a 1.5 mL microcentrifuge tube.
15. Centrifuge at 2000 rpm for 30 seconds.
16. Use 2 µL of the pellet and make a thin blood smear from both samples.
17. Fix the smears in methanol for 2 seconds and stain with Giemsa (section 6.2.6).

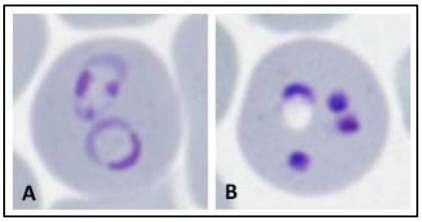
6.7. Microscopic examination to determine parasite survival rates

Three different thin blood smears must be stained and read per assay:

- "INI" smear to define the initial parasitemia at 0 hours
- "NE" smear to define the non-exposed parasitemia at 72 hours
- "DHA" smear to define the DHA-exposed parasitemia at 72 hours

1. At 100X magnification under immersion oil, count the number of infected RBCs containing viable parasites in a total of 10,000 RBCs (by estimating the number of RBCs per field or per grid). The smear must be homogenous: count areas on the slide containing 200-400 RBCs per field. Do not count areas at the edge of the smear since parasites will concentrate in these areas. Only viable parasites are scored (**see Figure 2**); vacuolated and pyknotic forms are not scored.
2. Determine the proportion of viable parasites (% survival) in NE and DHA slides.
3. Calculate:
    - Growth rate = NE/INI
    - Percentage survival (%) = (DHA/NE) x 100
4. Percentage survival values are interpretable if growth rate:
    - ≥ 1 for *ex-vivo* RSA
    - ≥ 1.5 for *in-vitro* RSA^{0-3h}

Figure 2. *Microscopic differentiation of viable (A) and dead (B) parasites 72 hours after drug exposure. 100X immersion, Giemsa stain.*



6.8. Flow cytometry analysis to measure parasite survival rates

All steps are performed at RT unless otherwise stated. **Measurement of parasite survival rates by flow cytometry can be carried out in addition to or in place of microscopic examination.**

6.8.1. Preparation and staining of P. falciparum samples

1. After centrifuging the sample at 2000 rpm for 30 seconds (subsection 6.6.3., step 15), transfer 20 µL of the packed cells to a new 1.5 mL microcentrifuge tube.
2. Suspend cells in 500 µL of flow cytometry sample wash solution and centrifuge at 2000 rpm for 30 seconds.
3. Aspirate supernatant and repeat wash two additional times.
4. Resuspend washed cell pellet in 180 µL of wash solution to make a 10% haematocrit cell suspension.
5. For each FACS sample (**see Table 3**), add 25 µL of cell suspension to a 1.5 mL microcentrifuge tube.
6. Add 25 µL of wash solution to the unstained sample.
7. Add 2 µL of 5X SG solution to 23 µL of wash solution to make a 0.4X SG solution and add it to the SG only sample.
8. Add 2 µL of 7.5 µM MT solution to 23 µL of wash solution to make a 0.6 µM MT solution and add it to the MT only sample.
9. Add 16 µL each of 5X SG solution and 7.5 µM MT solution to 168 µL of wash solution to make a 0.4X SG / 0.6 µM MT solution.
10. Add 25 µL of the 0.4X SG / 0.6 µM MT solution to the remaining doubly-stained samples.
11. Incubate all samples at 37°C in the dark for 30 minutes.
12. Add 500 µL of wash solution to each sample and centrifuge at 2000 rpm for 30 seconds.
13. Aspirate supernatants and repeat washes two additional times.
14. Resuspend each washed cell pellet in 500 µL of wash solution.
15. For each sample, add 150 µL of cell suspension to 1 mL of wash solution in a 5 mL round-bottom polystyrene tube.
16. Immediately proceed to run samples on a flow cytometer.

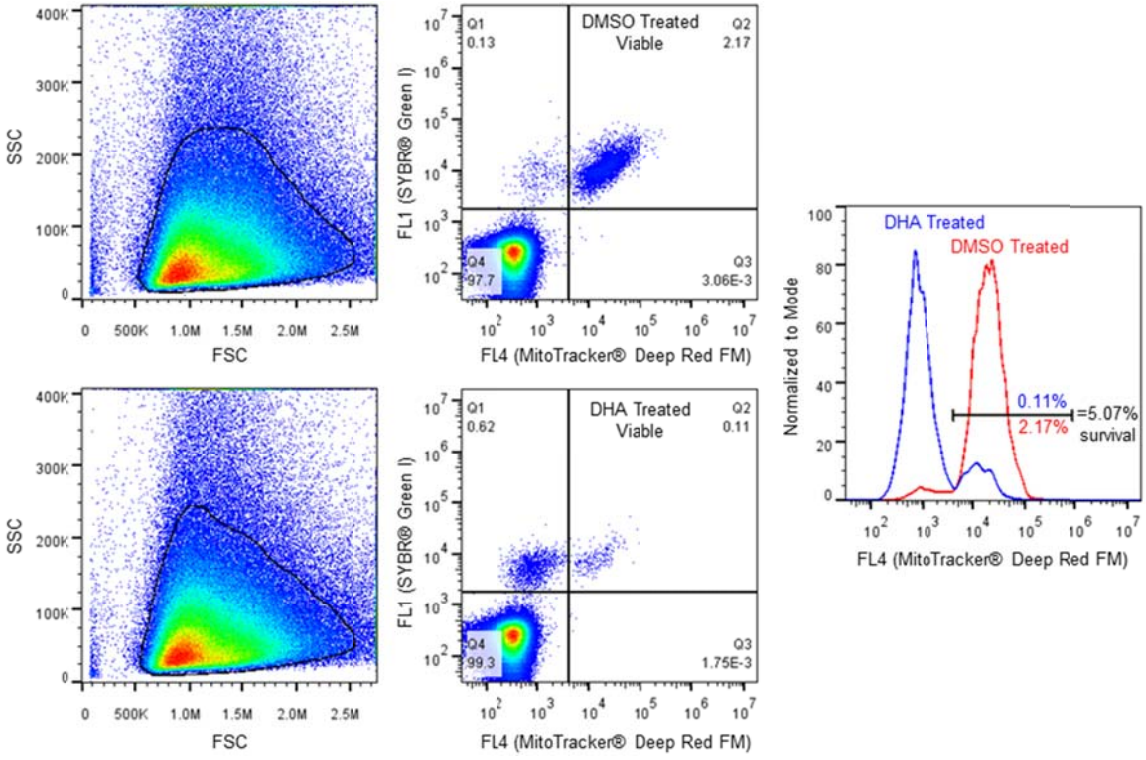
***Table 3**. Samples typically included in a single FACS experiment*

Sample	SYBR® Green I	MitoTracker® Deep Red FM	Purpose
DMSO treated cells - Unstained	No	No	Compensation Controls
DMSO treated cells - SG only	Yes	No	
DMSO treated cells - MT only	No	Yes	
DMSO treated cells	Yes	Yes	Triplicate samples to determine % survival
DMSO treated cells	Yes	Yes	
DMSO treated cells	Yes	Yes	
DHA treated cells	Yes	Yes	
DHA treated cells	Yes	Yes	
DHA treated cells	Yes	Yes	

6.8.2. Data collection and interpretation

1. Ensure that FACS samples are thoroughly mixed by briefly vortexing each tube.
2. On a calibrated flow cytometer, set up the machine parameters to collect 250,000 ungated events at a rate of approximately 2,500 events per second.
3. Ensure that both lasers are ready and that data will be collected in the FSC, SSC, FL1 (FITC), and FL4 (APC) channels.
4. Run the samples and save the data for processing according to Figure 3.
5. On a FSC by SSC plot, gate raw data to exclude debris and cell doublets.
6. On an FL4 by FL1 plot of the previously gated population, apply a quadrant gate to separate uninfected RBCs, pyknotic parasites, and viable parasites.
7. Apply this same quadrant gate to all DMSO treated and DHA treated samples.
8. Calculate:
    - Percentage survival (%) = (Q2 of DHA treated/Q2 of DMSO treated) x 100

Figure 3. *Flow cytometric **d**eterminati**o**n of perce**n**tage para**s**ite survival **l** 72 hours **a**fter drug exposure. **C**ells were stained wit**h** 0.2X SYBR® Green I a**n**d 0.3 µM **M**itoTracke**r**® Deep Red FM. **D**ata was co**l**lected on **a**n Accuri C6 flow cyto**m**eter (Bect**o**n Dickinson) and **a**nalyzed in FlowJo vX **(**Tree Star I**n**c.).*



7. References

1. Witkowski B, Amaratunga C, Khim N, Sreng S, Chim P, Kim S, Lim P, Mao S, Sopha C, Sam B, Anderson JM, Duong S, Chuor CM, Taylor WRJ, Suon S, Mercereau-Puijalon O, Fairhurst RM, Menard D. **Novel phenotypic assays for the detection of artemisinin-resistant *Plasmodium falciparum* malaria in Cambodia: in-vitro and ex-vivo drug-response studies.** *Lancet Infect Dis.* 2013; 13: 1043-1049.

2. Amaratunga C, Neal AT, Fairhurst RM. **Flow cytometry-based analysis of artemisinin-*resistant Plasmodium falciparum* in the ring-stage survival assay.** *Antimicrob Agents Chemother.* 2014; 58: 4938-4940.

Appendix A. Blood sample management for RSAs

patient's blood sample

maximum 24h post collection

WBC, plasma and anticoagulant removal

immediately

long term storage months or years (cryopreservation)

direct *in vitro* culture adaptation

delayed *in vitro* culture adaptation

ex vivo RSA

in vitro RSA

Appendix B. Schematic representation of RSAs