Malaria Journal

METHODOLOGY

Open Access

The extended recovery ring-stage survival assay provides a superior association with patient clearance half-life and increases throughput

Sage Z. Davis[1,2†], Puspendra P. Singh[1†], Katelyn M. Vendrely[1], Douglas A. Shoue[1], Lisa A. Checkley[1], Marina McDew-White[3], Katrina A. Button-Simons[1], Zione Cassady[1], Mackenzie A. C. Sievert[1], Gabriel J. Foster[1], François H. Nosten[4,5], Timothy J. C. Anderson[3] and Michael T. Ferdig[1*]

Abstract

Background: Tracking and understanding artemisinin resistance is key for preventing global setbacks in malaria eradication efforts. The ring-stage survival assay (RSA) is the current gold standard for in vitro artemisinin resistance phenotyping. However, the RSA has several drawbacks: it is relatively low throughput, has high variance due to microscopy readout, and correlates poorly with the current benchmark for in vivo resistance, patient clearance half-life post-artemisinin treatment. Here a modified RSA is presented, the extended Recovery Ring-stage Survival Assay (eRRSA), using 15 cloned patient isolates from Southeast Asia with a range of patient clearance half-lives, including parasite isolates with and without *kelch13* mutations.

Methods: *Plasmodium falciparum* cultures were synchronized with single layer Percoll during the schizont stage of the intraerythrocytic development cycle. Cultures were left to reinvade to early ring-stage and parasitaemia was quantified using flow cytometry. Cultures were diluted to 2% haematocrit and 0.5% parasitaemia in a 96-well plate to start the assay, allowing for increased throughput and decreased variability between biological replicates. Parasites were treated with 700 nM of dihydroartemisinin or 0.02% dimethyl sulfoxide (DMSO) for 6 h, washed three times in drug-free media, and incubated for 66 or 114 h, when samples were collected and frozen for PCR amplification. A SYBR Green-based quantitative PCR method was used to quantify the fold-change between treated and untreated samples.

Results: 15 cloned patient isolates from Southeast Asia with a range of patient clearance half-lives were assayed using the eRRSA. Due to the large number of pyknotic and dying parasites at 66 h post-exposure (72 h sample), parasites were grown for an additional cell cycle (114 h post-exposure, 120 h sample), which drastically improved correlation with patient clearance half-life compared to the 66 h post-exposure sample. A Spearman correlation of −0.8393 between fold change and patient clearance half-life was identified in these 15 isolates from Southeast Asia, which is the strongest correlation reported to date.

*Correspondence: ferdig.1@nd.edu
†Sage Z. Davis and Puspendra P. Singh authors contributed equally to this work
[1] Eck Institute for Global Health, Dept. of Biological Sciences, University of Notre Dame, Notre Dame, IN, USA
Full list of author information is available at the end of the article

Conclusions: eRRSA drastically increases the efficiency and accuracy of in vitro artemisinin resistance phenotyping compared to the traditional RSA, which paves the way for extensive in vitro phenotyping of hundreds of artemisinin resistant parasites.

Keywords: Ring-stage survival assay, Artemisinin resistance, *kelch13*

Background

Artemisinin (ART) resistance in malaria parasites is spreading through Southeast Asia and recent reports indicate that resistance has reached Southern Asia [1–3]. As artemisinin-based combination therapy (ACT) is the recommended course of treatment for uncomplicated malaria by the World Health Organization (WHO), the spread of ART resistance raises concerns for the future of malaria treatment [4]. The ability to track and understand ART resistance will be key in preventing global setbacks in malaria eradication efforts.

Measuring ART resistance is typically done in vivo using patient clearance half-life ($PC_{1/2}$), an assay that measures the linear decline of parasitaemia in patients after drug treatment [5–7]. Clinical ART resistance manifests as a delayed clearance of parasites from a patient's blood following treatment and is defined as a $PC_{1/2} \geq 5$ h [7]. While the $PC_{1/2}$ provides a method to track ART resistance in the field, it has drawbacks, namely the requirement for patients to meet a strict inclusion criteria and agree to hospitalization to measure the $PC_{1/2}$ [8]. To avoid this costly measure, in vitro measures of ART resistance have been developed. One of the most common in vitro measures of antimalarial drug resistance is the 50% inhibitory concentration (IC_{50}), which exposes parasites to serial dilutions of drug. However, delayed parasite clearance (as measured by $PC_{1/2}$) is not associated with a significant change in ART IC_{50} [8–10]. This is because later parasite stages (such as trophozoites and schizonts) are highly susceptible to ART, but early ring-stage ART resistant parasites (0–3 h) are able to survive pulses of ART. Therefore, the ring-stage survival assay (RSA) was developed to distinguish ART resistant parasites in vitro and to have a better correlation with $PC_{1/2}$ data than ART IC_{50}s [6, 8, 11].

The RSA has been the gold standard for measuring ART resistance in vitro, but it is a multi-step, laborious, and time-consuming assay that requires high volumes of very synchronized parasites. It is essential that the parasites are tightly synchronized in order to assay during the short window (0–3 h) that can differentiate ART resistant parasites from ART sensitive parasites. To do this, both a Percoll gradient and sorbitol are typically used [8], but several alterations have been attempted previously in other malaria assays to increase the throughput of the assay such as using both sorbitol and magnet columns [12], using syringe filters to select for merozoites [13], and using a dual layer Percoll gradient [14, 15]. Throughput of these various methods is dictated by the number of samples that can be simultaneously synchronized and prepared for processing. Another major bottleneck and source of variability in the final readout of the RSA is counting viable malaria parasites by microscopy [8, 11, 14]. To increase throughput, flow cytometry has become heavily utilized as an alternative to counting viable parasites by microscopy, removing hours of counting slides and human error [14, 16]. However, staining of cells for flow cytometry to detect viable parasites is time sensitive and requires samples to be prepared immediately after the 66 h incubation, which can be time consuming and inconvenient [14].

Despite these advances in the protocol, the RSA is still far from being both high-throughput and highly reflective of $PC_{1/2}$. Recently, Mukherjee et al. used the RSA to measure the percent survival of 36 culture-adapted parasites, but only showed a correlation with $PC_{1/2}$ data of 0.377, suggesting there is still significant room for improvement (Spearman's Rho, internal calculations based off of supplemental data) [17].

Here a modified RSA is presented: the extended recovery ring-stage survival assay (eRRSA). This modified RSA protocol utilizes a simple single layer Percoll synchronization, flow cytometry to determine the stage and parasitaemia for assay setup, a 96-well plate format for the assay, and a SYBR Green-based quantitative PCR (qPCR) method as the final readout. These modifications allow for an increased throughput in vitro experiment that better correlates with $PC_{1/2}$, allowing for improved segregation of resistant and sensitive parasites, as well as improved sorting of moderately resistant parasites. Further, efficiency improvements in the eRRSA allow for a higher throughput in vitro testing of ART resistance, accelerating our understanding of artemisinin resistance in the laboratory and providing a more accurate method to track the spread of resistance.

Methods

Parasite isolates

To evaluate the eRRSA methods, *P. falciparum* isolates with varying *kelch13* mutations and $PC_{1/2}$ were chosen. These isolates were derived from cloning by limiting dilution from patient samples. A total of 15 parasite

isolates were chosen, 9 of which have *kelch13* mutations (including one C580Y mutant, the most common *kelch13* mutation found in Southeast Asia currently), and a $PC_{1/2}$ distribution between 1.67 and 9.24. All 15 parasite isolates were isolated from patients on the Thailand-Myanmar border between 2008 and 2012. 3D7 was used as a control for comparison to the 15 Southeast Asian isolates (Table 1) [18, 19].

Parasite culture

Plasmodium falciparum isolates were cultured using standard methods in human red blood cells (RBC) (Biochemed Services, Winchester, VA and Interstate Blood Bank, Memphis, TN) suspended in complete medium (CM) containing RPMI 1640 with L-glutamine (Gibco, Life Technologies.), 50 mg/L hypoxanthine (Calbiochem, Sigma-Aldrich), 25 mM HEPES (Corning, VWR, 0.5% Albumax II (Gibco, Life Technologies.), 10 mg/L gentamicin (Gibco, Life Technologies) and 0.225% $NaHCO_3$ (Corning, VWR) at 5% haematocrit. Cultures were grown separately in sealed flasks at 37 °C under an atmosphere of 5% CO_2/5% O_2/90% N_2.

Percoll synchronization

Parasites were synchronized using a density gradient method as previously described with slight modifications [14, 15, 20]. Briefly, 350 µl of packed, infected erythrocytes at high schizogony (>50% schizonts) was suspended in 2 ml of RPMI. Cultures were layered over a single 70% Percoll (Sigma-Aldrich) layer in 1 × RPMI and 13.3% sorbitol in phosphate buffer saline (PBS) and centrifuged (1561×*g* for 10 min, no brake). The top layer of infected late stage schizonts was then removed and washed with 10 ml of RPMI twice. Cultures were then suspended in 2 ml of CM at 2% haematocrit and placed in culture flasks on a shaker in a 37 °C incubator for 4 h to allow for re-invasion.

Table 1 Parasite isolates used in this study

Isolate	*kelch13* mutation	$PC_{1/2}$	Location	Year
NHP1333	N458I	9.24	MKT	2011
NHP1455	R561H	8.02	MKT	2012
NHP1337	C580Y	7.83	MKT	2011
NHP4333	C538V	7.7	WPA	2008
NHP4373	WT	7.1	WPA	2008
NHP3035	M476I	6.16	MLA	2008
NHP4078	A675V	5.82	WPA	2008
NHP4201	WT	5.65	WPA	2008
NHP4106	WT	5.54	WPA	2008
NHP4673	E252Q	5.34	WPA	2010
NHP4748	WT	2.89	WPA	2011
NHP4072	WT	2.29	WPA	2008
NHP3032	K438N	2.17	MLA	2008
NHP4302	WT	1.98	WPA	2008
NHP1386	WT	1.67	MKT	2011

Overview of 15 parasite isolates used in this study. Patient clearance rates were calculated with [5]. Location of isolate collection and year of collection are as reported from Phyo et al. [19], Taylor et al. [28], and Cheeseman et al. [29]. Samples were all collected from clinics on the northwestern border of Thailand

MKT: Mawker Thai, MLA: Maela, and WPA: Wang Pha

Flow cytometry

Four hours after Percoll synchronization (unless noted otherwise), samples were measured by flow cytometry as previously described with slight modifications to determine parasitaemia [14, 16]. Briefly, 80 µl of culture and an RBC control incubated for at least 8 h at 2% haematocrit in CM were stained with SYBR Green I (SYBR) and SYTO 61 (SYTO) and measured on a guava easyCyte HT (Luminex Co.). Analysis was performed with guavaSoft version 3.3 (Luminex Co.). 50,000 events were recorded for both the RBC control and samples to determine relative parasitaemias.

eRRSA setup

Two hours post-cytometric quantitation (or 6 h after Percoll synchronization) samples whose stage composition was >70% rings as determined by flow cytometry were diluted to 2% haematocrit and 0.5% parasitaemia (unless otherwise noted), and 200 µl of culture was aliquoted into 6 wells of a flat bottom 96-well plate. Each treated and untreated sample had three technical replicates: RBC controls were aliquoted into 2 wells at 2% haematocrit and 200 µl. Three wells of parasites and 1 well of the RBC control were treated with 700 nM dihydroartemisinin (DHA) (Sigma-Aldrich); an additional 3 wells of parasites and 1 well of RBC control was treated with 0.02% dimethyl sulfoxide (DMSO) (ThermoFisher) as untreated controls. Parasites were incubated for 6 h, and then washed three times with 150 µl of RPMI to remove drug. Samples were then suspended in CM and placed back in the incubator. Sixty-six hours after drug removal, 20 µl of sample from each well was collected and frozen for qPCR amplification (72 h sample) without any media changes. Plates were then placed back in the incubator for another 48 h, after which 20 µl of sample was again collected and frozen for qPCR amplification (120 h sample).

qPCR Amplification

Ring-stage samples were quantified at 72 and 120 h post-drug treatment. qPCR was performed using the Phusion Blood Direct PCR kit (ThermoFisher, cat # F547L), supplemented with 1 × SYBR. Three microlitres of a 1:3 culture dilution was used in a 10 µl reaction and amplified using forward and reverse primers of the *pfcrt* gene. PCR amplification was measured using the fast mode of the ABI 7900HT, with a 20 s denaturation at 95 °C, followed

by 30 cycles of 95 °C for 1 s, 62.3 °C for 30 s, and 65 °C for 15 s (Additional file 1). Cycle threshold (Ct) values were calculated using the ABI SDS 2.4.1. Fold change ($2^{\Delta Ct}$) was calculated by determining the average ΔCt for the three technical replicates for the untreated and treated samples by applying the following equation:

$$Fold\ change = 2^{(average\ Ct\ of\ treated\ sample - average\ Ct\ of\ untreated\ sample)} \quad (1)$$

All statistics were performed and figures generated using GraphPad Prism version 8.2.1.

Results

Using a SYBR green-based quantitative PCR method to quantify the fold-change between treated and untreated samples

Percent proliferation is the standard RSA measurement to determine whether a parasite is artemisinin resistant or sensitive. This is calculated by dividing the percent parasitaemia in the treated (DHA) sample over the percent parasitaemia in the untreated (DMSO) sample. Parasitaemia is determined either by counting the number of viable and nonviable parasites using blood smears and microscopy or by flow cytometry. Determining parasitaemia with microscopy is cost effective and convenient but is also highly variable and time-consuming. Flow cytometry is typically very accurate; however, the process must begin upon reaching a time point, adding a substantial time investment at the point of sampling. To find another measurement of parasitaemia that could be automated and have less variability, qPCR on parasite genomic DNA was tested. A standard curve of percent parasitaemia (as measured by flow cytometry) shows excellent inverse correlation with Ct values as measured by qPCR (Fig. 1). To quantify the difference between treated and untreated final RSA samples, fold change ($2^{\Delta Ct}$) was calculated according to Eq. 1.

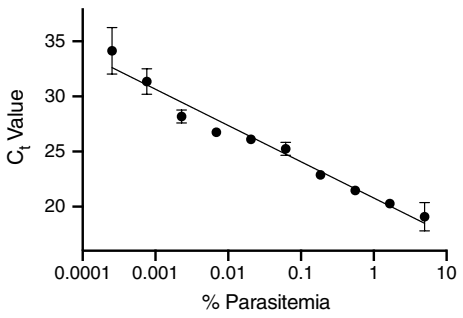

Fig. 1 qPCR standard curve for detecting parasitaemia of *P. falciparum* infected RBCs. A standard curve of samples ranging from 0.0001% parasitaemia to 9% parasitaemia were measured using qPCR amplification on the ABI 7900HT. Ct values were calculated based on three technical replicates using the ABI SDS 2.4.1. Ct value is inversely related to percent parasitaemia ($R^2 = 0.9699$) and therefore can be used as a measurement of percent parasitaemia in final RSA samples

RSA readout at 120 h provides superior differentiation between sensitive and resistant isolates

The standard RSA determines parasite viability at 72 h after drug treatment. However, a common problem in the final readout (using either microscopy, flow cytometry, or qPCR) is the difficulty in differentiating between pyknotic (nonviable) parasites and viable parasites 72 h after drug treatment. It is also difficult to measure viable parasitaemia when it can be as low as 0.01% (or even 0% in some cases), especially when measuring ART sensitive parasites [21]. To address these issues, the time to readout was extended by an additional intraerythrocytic development cycle (48 h); previous methods have extended the time to readout by 24 h in specific cases [13, 22]. This extension was added to both allow parasites an additional expansion cycle, creating larger differences to distinguish resistant and sensitive isolates, and to allow erythrocytes to clear pyknotic parasites. This additional cycle provides a much greater separation between resistant and sensitive parasite isolates (Fig. 2).

Assay setup conditions have a substantial impact on RSA outcomes

The RSA is a growth assay targeted at a very narrow window of the parasite intraerythrocytic development cycle. In order to maximize the precision of the assay, the growth and the timing of the target window were carefully optimized. First, as it has been established that growth rates can vary based on parasitaemia, the effect of varying starting parasitaemias on RSA outcome was observed [23]. RSA was performed on three parasite isolates (3D7, 1337, and 4673) at varying starting parasitaemias as determined by a microscopist, and samples were collected at 72 h and 120 h (Additional file 2A, B, respectively). For the three parasite isolates tested, 3D7 was the ART sensitive control and 1337 and 4673 were two ART resistant parasite isolates as determined by their $PC_{1/2}$ values (1337 $PC_{1/2} = 7.84$ and 4673 $PC_{1/2} = 5.34$). The 0.25% starting parasitaemia showed the most distinguishable phenotype between the ART sensitive 3D7 control and the two ART resistant isolates at 120 h (Additional file 2B). It was noted internally that determinations of parasitaemia by microscopy varied widely and underestimated parasitaemia compared to flow cytometry, likely due to the difficulty of correctly identifying new invasions. A comparison of RSA results from isolates set up at 0.25% parasitaemia determined by microscopy and 0.5%

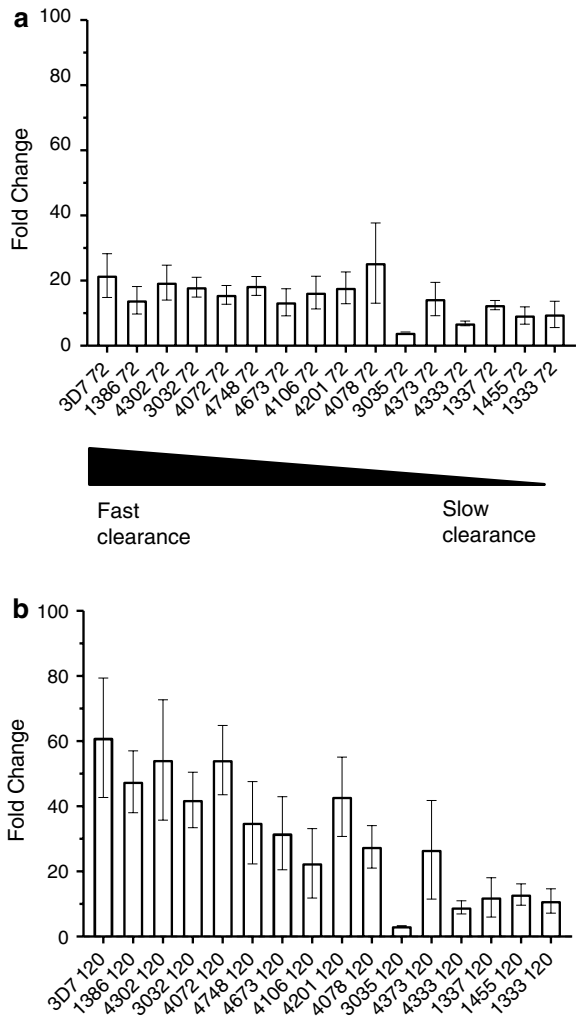

Fig. 2 Comparison of 72 h and 120 h perturbations. Parasites were set-up using the eRRSA protocol: 0.5% parasitaemia at early ring-stage and 700 nM DHA was applied and washed off after 6 h. Samples were collected at **a** 72 h post-drug treatment and at **b** 120 h post-drug treatment. Parasites are ordered from the smallest $PC_{1/2}$ (least resistant) to the largest $PC_{1/2}$ (most resistant) from left to right (with the sensitive control, 3D7 on the far left). Sensitive and resistant parasites were more distinguishable based on their 120 h post-drug treatment sample fold changes

parasitaemia determined by flow cytometry showed no difference between the two (Additional file 3). As a result, in subsequent setups starting parasitaemia was determined by flow cytometry and was normalized to 0.5% parasitaemia.

A key factor in the RSA is applying the drug treatment in the tight 0–3 h window of the parasite life cycle that can differentiate ART resistant parasites from ART sensitive parasites. Therefore, the time from Percoll synchronization to drug treatment was also varied to find the optimal time for drug treatment. The same three parasite isolates, (3D7, 1337, and 4673), were set up and treated with 700 nM DHA 4 h, 6 h, 8 h, or 10 h after Percoll synchronization and samples collected at 72 h and 120 h post-drug treatment (Additional file 2C, D, respectively). All parasites were set up at a starting parasitaemia of 0.5% as measured by flow cytometry. The time that resulted in the most consistent and distinguishable phenotypes between the ART sensitive and resistant isolates was 6 h post-Percoll synchronization. Controlling these factors resulted in a more consistent and reproducible RSA phenotype.

Defining the eRRSA as an improvement over the standard RSA

Based on the previously described optimizations, the eRRSA is defined as a single layer Percoll synchronization, flow cytometry measurement of parasite parasitaemia and stage prior to assay setup, 700 nM DHA treatment of 200 µl of parasites at 2% haematocrit and 0.5% parasitaemia in 96-well plates that are set up at 6 h post-Percoll synchronization, drug washed off 6 h after application, and samples for qPCR readout collected at 120 h post-drug treatment (Additional file 4). This extension to 120 h was added to both allow parasites an additional expansion cycle to recover from drug treatment, creating larger differences to distinguish resistant and sensitive isolates, and to allow erythrocytes to clear pyknotic parasites. This protocol provides the most consistent and higher throughput results. These modifications made to the standard RSA are a new in vitro ART resistance phenotyping method: the Extended Recovery Ring-stage Survival Assay (eRRSA).

eRRSA correlates better with $PC_{1/2}$ than RSA

The RSA was introduced as an in vitro method that better captures the gold standard for in vivo ART resistance, $PC_{1/2}$, than the traditional IC_{50}. For a new assay to be relevant, it should perform at least comparably to the existing standard. Therefore, the eRRSA was used to assay 15 isolates from Southeast Asia with varying known $PC_{1/2}$ and *kelch13* mutations collected between 2008 and 2012 (Table 1). NHP1337 was used as a resistant parasite isolate control and 3D7 was used as a sensitive parasite control. Three biological replicates were collected (each with three technical replicates) for each isolate and collected samples at 72 h and 120 h post-drug treatment and compared the viability of treated and untreated parasites at each stage.

The fold change data was then compared to $PC_{1/2}$: at the 72 h timepoint across 15 isolates, the eRRSA has a Spearman correlation coefficient of −0.6071 (Fig. 3a). This is comparable to other RSA correlations in the field, showing that the improvements made to the RSA

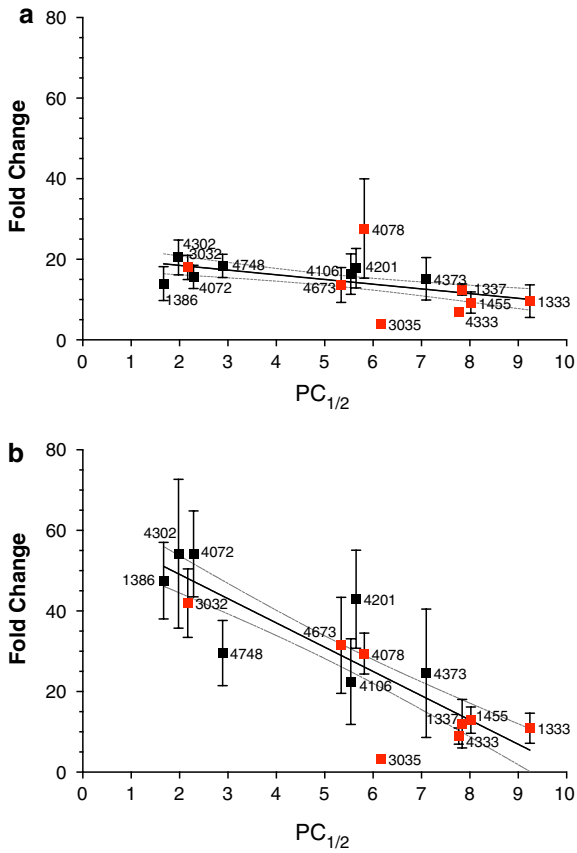

Fig. 3 120 h eRRSA increases correlation with $PC_{1/2}$ compared to 72 h. **a** 15 isolates from Southeast Asia with varying $PC_{1/2}$ were assayed using the eRRSA. 72 h post-drug treatment samples were measured to give a fold change for each isolate and those fold changes were correlated with each isolate's $PC_{1/2}$ (Spearman r = −0.6071). Isolates with red boxes are *kelch13* mutants and the 95% confidence interval around the best-fit line ($y = -6.025x + 61.14$) is denoted with dotted lines. **b** The same 15 isolates were assayed using the eRRSA and 120 h post-drug treatment samples were measured to calculate a fold change for each isolate. The fold changes were correlated with $PC_{1/2}$ (isolates marked with red boxes are *kelch13* mutants and the 95% confidence interval of the best-fit line is denoted with dotted lines) (Spearman r = −0.8393); the 120 h eRRSA samples showed an increased correlation with $PC_{1/2}$ compared to the 72 h samples

protocol do not significantly affect the outcome while increasing efficiency and ease of the assay [8, 17]. The 120 h correlations, however, improved Spearman correlation between fold change and $PC_{1/2}$ to −0.8393 (Fig. 3b).

Traditional RSA uses a value of ≥1% parasite survival to indicate ART resistance [8]. To determine a value for ART resistance as measured by the eRRSA, the best-fit line for the 120 h post-drug treatment (Fig. 3b) was used to calculate the fold change when $PC_{1/2}$ is 5 (given that $PC_{1/2} \geq 5$ h defines clinical ART resistance [7]. Therefore, a fold change of 30 or less defines an ART resistant parasite by eRRSA.

Discussion

With the gradual spreading of artemisinin resistance throughout Southeast Asia, it is imperative that resistance can be accurately measured both in the field and in the lab. To date, the RSA has been the golden standard for in vitro measurement of artemisinin resistance. In the clinic, $PC_{1/2}$ is the standard for in vivo measurement of artemisinin resistance. The $PC_{1/2}$ comprises contributions by both the human host and the parasite. Because the RSA is an in vitro measurement of only the parasite component, the RSA cannot perfectly correlate with $PC_{1/2}$ [24–27]. Despite this, there is a need for a more accurate, higher throughput in vitro measurement alternative to the current RSA to accelerate understanding of artemisinin resistance. The eRRSA was developed for this purpose and this study shows that it can outperform the RSA in both accuracy and efficiency.

A major bottleneck and source of variability in the RSA is the final readout to determine the ratio of viable parasites in the treated and untreated cultures. RSA uses microscopy or flow cytometry as the final readout, while the eRRSA uses qPCR. When using microscopy to compare viable and nonviable parasites, the presence of the ring-like structure of healthy, viable parasites are compared to the collapsed, nonviable parasites which can be highly subjective and time consuming, as shown by the original RSA paper which required two to three microscopists [14]. Flow cytometry utilizes the DNA stain SYBR and the mitochondrial stain MitoTracker red to differentiate between viable and nonviable parasites. This eliminates the requirement of microscopists and drastically decreases the labor required to determine percent proliferation of parasites [14, 16]. However, staining of cells for flow cytometry is time sensitive and must be done immediately following the end of the RSA (at 72 h), which can limit flexibility and lengthens an assay that already demands long hours. qPCR measure viable parasites solely by concentration of genomic material, comparing the efficiency of parasites to proliferate post-artemisinin perturbation. Here, the efficacy of qPCR is demonstrated as a readout for proliferation in a survival assay context. The use of qPCR allows for both smaller sample sizes and a delayed readout, rendering the protocol easier and more precise.

The eRRSA measures the difference in genomic content between treated and untreated malaria parasites at 120 h post perturbation, a full 48 h (or a full life cycle) after the RSA collections. The results demonstrate that

by allowing parasites an extra life cycle to recover, the differences between resistant and sensitive parasites are made even more drastic, suggesting the extra lifecycle for recovery helps further differentiate viable and nonviable parasites. With an extra life cycle for recovery from drug treatment, the eRRSA measures recovery of the parasites, rather than the survivability of the parasites after treatment, and provides a more consistent readout compared to other publications (Additional file 5).

The demonstration of the effect of various setup conditions on the final outcome required an optimization of these parameters in the eRRSA. With no sorbitol synchronization required and only one single layer Percoll synchronization to select schizonts, parasites are synchronized easier, quicker, and closer to the ring-stage so that they can be set up in the assay sooner to avoid losing their synchronization. The variability in assay outcome caused by varying the delay between synchronization and treatment is likely due to the short window (0–3 h) that differentiates ART resistant parasites from ART sensitive parasites. The eRRSA assay is set up 6 h post-Percoll, which allows for a high percentage of early ring-stage, tightly synchronized parasites. Using flow cytometry for parasitaemia measurements post-Percoll synchronization allows for rapid and accurate parasitaemia determination and staging for many parasites at once at the setup of the assay, which is an essential factor for the results of both RSA and eRRSA. Starting parasitaemia has a substantial effect on growth throughout the assay; by using a lower starting parasitaemia (0.5%) compared to the in vitro RSA, the eRRSA has a lower volume and parasitaemia requirement while also allowing for more precise measurement of growth with and without drug treatment. Lower culture volume requirements permit the use of 96-well plates, which uses less reagents, time, and space.

The RSA is a multi-step and time-consuming assay. Even with the optimizations of the eRRSA, the 4 h required to wait until schizonts have reinvaded after single layer Percoll synchronization to obtain 0–3 h ring-stage parasites and the 6 h of drug incubation time cannot be avoided. However, by automating aspects of the assay that have a certain run time regardless of how many samples are being assayed (e.g., flow cytometry in a 96-well plate to set-up parasites and qPCR as the final readout), running larger numbers of samples in parallel does not add a significant amount of time to the assay. In addition to automation, decreasing the culture volumes (by using a 96-well plate) and using a quick and tight synchronization method (single layer Percoll) makes it possible for one researcher to set-up 12–15 parasites samples with technical replicates in a ~ 12 h day (Additional file 4).

Finally, it was demonstrated that the eRRSA shows superior correlation with the clinical phenotype $PC_{1/2}$. As ART resistance is currently presenting as a continuous phenotype, the ability to accurately determine intermediate phenotypes is critical in understanding ART resistance and identifying contributing genotypes beyond *kelch13* propeller mutations. The efficiency of the eRRSA makes it an excellent replacement for the traditional RSA in any study of ART resistance requiring accuracy and higher throughput. The 15 cloned parasites examined include three which lack *kelch13* mutations but show $PC_{1/2} > 5$. These include one clone (NHP4373) with $PC_{1/2} = 7.1$. Interestingly, these clones also show high eRRSA values, confirming their ART resistant status. These results provide further support that ART resistance may result from mutations elsewhere in the parasite genome, or perhaps from non-coding regulatory changes controlling *kelch13* activity [17].

Conclusions

The eRRSA method described here provides a more robust in vitro representation of $PC_{1/2}$ while also providing vastly improved throughput. Widespread adaptation of the eRRSA should significantly accelerate our understanding of artemisinin resistance, allowing for both high throughput surveillance of the spread of resistance and for the precise phenotyping necessary to uncover complex genetic contributors to resistance.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12936-020-3139-6.

Additional file 1. Table of primer and cycling conditions used for quantitative PCR. The forward and reverse primer sequences of the *pfcrt* gene used in this study and the cycling conditions used in the fast mode of the ABI 7900HT. Primer sequence was created using Integrated DNA Technologies' (IDT) qPCR primer design software. Using fold change as the readout for qPCR (comparing the treated sample to the untreated sample of the same parasite isolate) avoids any amplification bias between different parasite isolates.

Additional file 2. Effects of assay modification on final assay readout. (A) The starting parasitaemia for the assay was set at 0.25%, 0.5%, or 1% (as measured by microscopy) for three different parasite isolates (3D7, 1337, and 4673) and the fold change between the treated (DHA) and untreated (DMSO) samples was measured with three biological replicates (each with three technical replicates) at 72 h post-drug treatment and (B) at 120 h post-drug treatment. After determining that a 0.25% parasitaemia measured by microscopy was equivalent to 0.5% parasitaemia measured by flow cytometry (C), the starting parasitaemia was determined using flow cytometry and set to 0.5% and the time from Percoll synchronization to drug (DHA or DMSO) application was varied (4 h, 6 h 8 h, or 10 h) for the three different parasite isolates (3D7, 1337, and 4673) and the fold change between treated and untreated samples was measured with three biological replicates (each with three technical replicates) at 72 h post-drug treatment and (D) 120 h post-drug treatment. The effect of varying starting parasitaemia and time to drug treatment post-synchronization on fold change was calculated using a one-way ANOVA. Statistically significant differences between treatments are reported on the corresponding figures.

Additional file 3. Comparison of microscopy vs flow cytometry to calculate starting parasitaemia. Fold change data for two parasite lines (3D7 and 1337) used as controls in eRRSA show no significant difference ($p = 0.21$ and 0.20, respectively) between a starting parasitaemia of 0.25% by microscopy (MS) and a starting parasitaemia of 0.5% determined by flow cytometry (FC). P-values were calculated using an unpaired t-test with Welch's correction.

Additional file 4. Summary of modifications for the eRRSA compared to the standard RSA. A timeline comparing the various steps and the estimated time needed for each step of the standard WWARN RSA [14] (left) and the eRRSA (right). There are limiting steps in both the eRRSA and RSA (marked with orange arrows), but the eRRSA allows for more parasite isolates with more biological replicates to be done in less time compared to the standard RSA.

Additional file 5. Comparison of previously published correlation rates of RSA results with $PC_{1/2}$. Previous publications where $PC_{1/2}$ and RSA results on the same samples were reported are listed here. If RSA results were available, then the Spearman correlation between $PC_{1/2}$ and RSA was calculated. If the Spearman correlation was not provided, it was internally calculated and is marked with an*.

Abbreviations
RSA: Ring-stage survival assay; eRRSA: Extended recovery ring-stage survival assay; DMSO: Dimethyl sulfoxide; ART: Artemisinin; ACT: Artemisinin-based combination therapy; WHO: World Health Organization; $PC_{1/2}$: Patient clearance half-life; qPCR: Quantitative polymerase chain reaction; RBC: Red blood cell; CM: Complete media; PBS: Phosphate buffer saline; SYBR: SYBR Green I; SYTO: SYTO 61 red fluorescent nucleic acid stain; DHA: Dihydroartemisinin; Ct: Cycle threshold; QTL: Quantitative trait loci.

Acknowledgements
We would like to acknowledge members of the Ferdig lab for helpful discussions.

Authors' contributions
SZD, LAC, DAS, and MTF conceived and designed the experiments. PPS, LAC, DAS, GJF, and MACS performed the experiments, analysed the data, and created figures. SZD, KMV, LAC, and MTF wrote the manuscript. FHN, MM and TJCA contributed materials/reagents/parasites. All authors read and approved the final manuscript.

Funding
Funding for this work was provided by NIH grant P01 AI127338 to MTF. TJCA and MM were funded by R37 AI048071. SMRU is part of the Mahidol Oxford University Research Unit supported by the Wellcome Trust of Great Britain.

Availability of data and materials
Data can be made available upon request to the corresponding author.

Ethics approval and consent to participate
Ethical approval for the use of human blood in this study was granted by the Institutional Review Board of the University of Notre Dame. All of the blood used for the in vitro culturing of parasites was obtained from healthy adult volunteers and drawn by trained personnel from Interstate Blood Bank.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1] Eck Institute for Global Health, Dept. of Biological Sciences, University of Notre Dame, Notre Dame, IN, USA. [2] Molecular, Cell, and Systems Biology Department, University of California Riverside, Riverside, CA, USA. [3] Texas Biomedical Research Institute, San Antonio, TX, USA. [4] Shoklo Malaria Research Unit, Mahidol-Oxford Tropical Medicine Research Unit, Mahidol University, Mae Sot, Thailand. [5] Centre for Tropical Medicine and Global Health, Nuffield Department of Medicine Research Building, University of Oxford Old Road Campus, Oxford, UK.

Received: 9 December 2019 Accepted: 24 January 2020
Published online: 31 January 2020

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