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Malaria Vaccine Development: Focusing Field Erythrocyte Invasion Studies on Phenotypic Diversity:

The West African Merozoite Invasion Network (WAMIN)

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Abstract

Erythrocyte invasion by *Plasmodium falciparum* merozoites is an essential step for parasite survival and proliferation. Invasion is mediated by multiple ligands, which could be promising vaccine targets. The usage and sequence of these ligands differs between parasites, yet most studies of them have been carried out in only a few laboratory-adapted lines. To understand the true extent of natural variation in invasion phenotypes and prioritize vaccine candidates on a relevant evidence base, we need to develop and apply standardized assays to large numbers of field isolates. The West African Merozoite Invasion Network (WAMIN) has been formed to meet these goals, expand training in *Plasmodium* phenotyping, and perform large-scale field phenotyping studies in order to prioritize blood stage vaccine candidates.

The Need for a Globally Effective Malaria Vaccine

Malaria remains one of the leading contributors to global infectious disease-related mortality. With drug resistance an ever-recurring problem, efforts to develop an effective *Plasmodium falciparum* vaccine remain high on the global research agenda [1]. No licensed

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malaria vaccine currently exists, although the circumsporozoite protein-based candidate RTS,S was recommended for licensing by the European Medicines Agency in July 2015. However, protection is achieved in only approximately one-third of those vaccinated and is short lived [2–4], so a more effective and comprehensive second-generation vaccine is clearly needed. While many potential targets exist in the >5000 gene *Plasmodium falciparum* genome, it will be crucial that validation of all potential targets takes into account the considerable natural genetic and phenotypic variation found in *P. falciparum* clinical isolates. Advances in whole genome sequencing are now rapidly expanding the study of genetic variation in clinical isolates, but our understanding of their phenotypic diversity lags far behind. In this opinion article, we summarize the opportunities and challenges of erythrocyte invasion ligands as vaccine targets, and describe how some of the challenges could be overcome by systematic studies in endemic countries. To facilitate such studies, we outline recommendations and areas for further investigation to allow a standardized field-based invasion assay to be developed. Such an assay, together with a network of trained and experienced laboratories, will establish a unique platform for vaccine validation in West Africa.

Erythrocyte Invasion as an Attractive Vaccine Target

The erythrocytic stage of the parasite in general, and the process of erythrocyte invasion in particular, are attractive targets for the development of vaccines. Clinical symptoms are caused only by this stage of the life cycle, and the parasite relies on successful replication in erythrocytes to generate the transmissible sexual stage forms [5]. As a result, effectively inhibiting the parasite from invading erythrocytes will likely prevent clinical disease and also impact transmission. The invasion process begins with the attachment of a merozoite to the surface of an erythrocyte through weak interactions between merozoite surface proteins and erythrocyte proteins (Figure 1). Attachment is followed by stronger, more specific interactions mediated by parasite ligands, which are released from the merozoite's rhoptries and micronemes, and are thought to interact with one or more erythrocyte surface proteins. The ligands involved in attachment primarily belong to two major families of proteins, the erythrocyte binding-like (EBL) family including the erythrocyte binding antigens EBA-140, EBA-175, EBL-1, and EBA-181 and the reticulocyte binding-like homologs (RH) including PfRH1, PfRH2a, PfRH2b, PfRH4, and PfRH5 [6,7]. These parasite ligands each bind to unique erythrocyte surface receptors, and can vary both in expression and sequence between different parasite strains. As a result, different strains can invade erythrocytes using alternative receptors, frequently referred to as alternative invasion pathways [8–10].

Perhaps the most prominent example of alternative pathways involves interactions between specific parasite EBL and RBL proteins and sialic acid (SA) residues on erythrocyte surface glycoprotein proteins A, B, and C [7]. While SA-dependent invasion is common, many parasite strains are also capable of invading erythrocytes lacking SA, by utilizing alternative receptors such as complement receptor 1 (CR1) [11–13]. As well as alternatives operating at the same step of invasion, different invasion ligands may also operate at different temporal steps. The PfRH5 ligand is one example that may play a role later in the invasion process, forming tight contacts with the erythrocyte through interaction with the Ok blood group antigen, basigin, and triggering local calcium release [14]. So far, PfRH5 is the only parasite

ligand of the EBL and RBL families that has been shown to have an essential role for erythrocyte invasion by all strains of *P. falciparum* [15–17].

Phenotypic Diversity in Erythrocyte Invasion

With the exception of PfRH5, which as noted above may operate at a distinct step during invasion, almost as soon as invasion ligands have been identified to play a role in invasion, strains have been identified, or generated through genetic manipulation, that can invade in the absence of that specific ligand [7,17–21]. This presents a major challenge for the development of a vaccine targeting erythrocyte invasion. If there are multiple ligands that are differentially deployed in different parasite strains, there must be a degree of redundancy, and to be effective a vaccine must cover the entire interaction space. In addition, significant sequence polymorphism has been identified in the receptor binding domains of invasion ligands [7]. This diversity is thought to be a potential mechanism in the evasion of immunity and/or the use of alternative invasion pathways [22]. Understanding the extent and causes of phenotypic diversity in erythrocyte invasion will be crucial if we are to exploit our knowledge of invasion mechanisms for vaccine development.

Researchers have been trying to classify phenotypic diversity in invasion phenotypes for decades. The most common approach is to define invasion pathways by using the known sensitivities of erythrocyte receptors to particular enzyme treatments, particularly neuraminidase, which removes SA residues, and the proteases trypsin and chymotrypsin, which cleave particular sites on peptide backbones. For example, glycophorin A is trypsin-sensitive but resistant to chymotrypsin, glycophorin B is removable by chymotrypsin [7], CR1 is sensitive to both trypsin and chymotrypsin [7], and basigin is resistant to both enzymes [15]. The individual and combinatorial use of these three enzymes has therefore been frequently used in erythrocyte invasion studies to phenotypically categorize malaria parasites. While broadly available and therefore more easily used, enzymes have the disadvantage of affecting multiple receptors at the same time. The sensitivity of parasites to inhibition by antibodies specific for different parasite ligands or receptors is a potentially more specific approach [15,17,23,24], although it too has limitations, particularly in the availability of sufficient quantities of standardized reagents to perform broad comparative studies. Additional powerful approaches requiring specific reagents include using mutant erythrocytes lacking specific receptors or knocking-down receptors using small hairpin RNA (shRNA) in stem cell derived erythrocytes, although the small scales of the culture and diversity in the age of cultured erythrocytes is a challenge in the latter system [15,25–28]. Recently, complement was identified as an additional factor that can influence the degree of inhibition of anti-*Plasmodium* antibodies, and incorporating these observations into studies of phenotypic diversity may also be important [29]. Beyond diversity in ligand sequence or usage, fundamental measures of parasite invasion include the parasite multiplication rate (PMR) – the efficiency of reinvasion after a single cycle – and selectivity index (SI) – the quantitative assessment of single versus multiply infected cells – which can also vary considerably between parasite isolates [30–32].

There are therefore multiple methods that can be used to determine the dependence of parasite strains on specific ligand–receptor interactions. It is important to emphasize,

however, that the vast majority of these methods have been applied primarily to only on a few laboratory-adapted *P. falciparum* strains, which in some cases have been maintained in culture in the laboratory for several decades since they were first isolated from patients. A critical question therefore is how representative are these laboratory strains of the *P. falciparum* isolates that are circulating in populations around the world today: the populations that would need to be inhibited by a vaccine.

Investigations of Erythrocyte Invasion Phenotypes in Field Isolates

To address this, pioneering and technically challenging studies have been performed to phenotype erythrocyte invasion in field isolates. At the time of this article, erythrocyte invasion phenotypes have been measured in 13 independent studies on 536 *P. falciparum* clinical isolates across ten different countries (Figure 2A); the overwhelming majority of these isolates were from patients in West Africa (Figure 2B; 437 isolates, 81.5% of the total). These studies measured invasion phenotypes primarily by measuring the sensitivity of invasion to enzyme treatments of erythrocytes by neuraminidase, trypsin, and chymotrypsin. Strikingly, all studies indicate a wide diversity of phenotypes existing at each endemic location, regardless of transmission intensity or endemicity [12,17,23,31–40].

However, while some broad principles can be drawn by comparing these previous studies, precise comparisons among them are limited by their use of very different protocols [41], even among the studies conducted in West Africa (Figure 2B). Although some variation in the distribution of phenotypes are apparent between studies, such as the extreme sensitivity to trypsin observed in Senegal but a more moderate sensitivity observed in Gambia [38], it is not straightforward to determine whether these reflect real biological differences in parasite phenotypes between countries or locations, or technical differences in methods or reagents used. Potential sources of variation may include whether parasites are tested directly after collection (*ex vivo* or ‘fresh’) or are cryopreserved, differences in sample collection and cryopreservation or thawing protocols, differences in parasite culture times, differences in enzyme suppliers and variation of enzyme activity between batches, concentrations of enzymes, enzyme storage conditions, and different methods used to quantitate parasitemia.

Comparison of samples from multiple populations together within the same laboratory can eliminate some of this variation, as has been done for a subset of samples from three different countries [40]. However, this approach faces a logistic limit in terms of the overall number of samples that a single laboratory can assay. For well-powered population-based association studies or rigorous preclinical assessment of novel vaccine targets, it might be necessary to phenotype a few thousand individual phenotypes, which is beyond the capacity of any one existing laboratory. In this case, it will be important to harmonize and standardize phenotyping methods, taking into consideration the reality of local settings in which assays will be conducted. The challenges are global, and may be tackled initially by developing more effective means of understanding erythrocyte invasion in West Africa, where the vast majority of field isolates have been studied to date.

West Africa Merozoite Invasion Network

Given these challenges, significant advances will require an active network of collaborating research groups that can contribute comparable data and materials, just as large networks using standardized approaches have revolutionized the study of common human genetic diseases through genome-wide association studies (GWAS). The West Africa Merozoite Invasion Network (WAMIN) has been formed in response to this need. WAMIN is a small group of scientists who are actively involved in erythrocyte invasion studies in the highly endemic region of West Africa, and have committed to working together to standardize protocols, train and certify young scientists, and share reagents and data. WAMIN held its inaugural workshop entitled ‘Targeting Malaria: *Plasmodium falciparum* Invasion Assays’ at the Laboratory of Bacteriology–Virology at Aristide Le Dantec Hospital in Dakar, from June 8 to June 13, 2015. The meeting brought together investigators in the field of *P. falciparum* erythrocyte invasion to share ideas on assay procedures and to train young scientists on methods for studying invasion mechanisms. Twelve trainees from Senegal, the Gambia, Mali, Burkina Faso, Nigeria, and Ghana were taken through 5 days of intensive laboratory work and theoretical discussions to better understand and perfect invasion assay techniques. It also provided an opportunity to establish parameters for standardization and develop a harmonized protocol.

The Need for Standardized Phenotyping Protocols

Phenotyping erythrocyte invasion is a complex process, and unlike drug resistance phenotyping requires manipulation of both uninfected erythrocytes and parasites. Invasion assay protocols are long and complicated, with an extensive list of features that could potentially need to be standardized across different laboratories (Figure 3). So far, there have been few attempts to phenotype using standardized approaches in multiple locations. WAMIN partner Gordon Awandare at the West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), at the University of Ghana used standardized methods for sample collection, processing and storage, of parasites from different transmission areas in Ghana, and then analyzed them for their invasion phenotypes through a standardized assay procedure in one laboratory. These assays demonstrated differences between parasite isolates, were powerful enough to resolve phenotypic variation between three transmission areas, and suggested that SA-independent invasion levels correlated with parasite transmission intensities [17]. The GENINVADE project led by WAMIN partner David Conway at the London School of Hygiene and Tropical Medicine (LSHTM) cryopreserved parasites from three endemic areas in different countries (Ghana, Guinea, and Senegal) and then phenotyped them in a single laboratory using a standardized protocol, with the investigators blinded to the sample source. This approach also indicated some differences in invasion phenotypes between the three countries [40]. However, both studies showed that the diversity of clinical isolate phenotypes within each endemic area was greater than the differences between the areas, emphasizing the need for large sample sizes to test for significant differences. Additionally, there were concordant phenotypes for the small number of isolates that were tested in both studies, illustrating the need for further standardization.

The WAMIN workshop and this opinion article are starting points in the drive to develop a single standard operating procedure (Figure 3), which should give the same results no matter when or where they are applied. Even though more than 500 successful (and many more unsuccessful) field invasion assays have been performed, there is currently no universally accepted standard for any element of the protocol. Some key technical elements that would make field invasion assays more standardized and comparable are easily defined. Box 1 summarizes a list of those elements; all WAMIN partners have committed to incorporating these recommendations into their research, and urge others to do the same. In other cases, elements of the invasion phenotyping protocol that could impact variability are harder to standardize, and we currently lack quantitative data on what impact these variables might have. The Outstanding Questions box identifies a list of variables that need further investigation before a final standardized protocol can be developed.

Large-Scale Studies of Invasion to Drive Vaccine Target Prioritization

A standardized protocol would enable large-scale studies, which could investigate key unanswered questions in invasion biology. Despite extensive work with established laboratory isolates, our understanding of the genetic basis of invasion phenotypes in the field remains extremely fragmentary. The peculiarities of transmission intensity, coupled with potential differences in population structure between endemic regions, suggest that we now need to increase our coverage of new populations for both phenotypic and genetic analysis, through a comprehensive long-term multipopulation investigation across endemic regions. Building on experience in the ongoing GENINVADE and WACCBIP studies [17,40], WAMIN proposes to synergize and standardize studies and approaches and build the necessary technical and skills capacity to compare invasion phenotypes across populations in a systematic manner. This will include standardized methods for sample collection, processing and storage of parasites from different transmission areas across several endemic countries in West Africa, and invasion phenotyping using the finalized standard operating procedure (SOP) in multiple endemic countries. Important considerations for this development process include the capacity of the laboratories in the endemic countries to produce reliable data, challenges, and risks in transporting large numbers of samples across countries, and long-term sustainability of the approach. Coupling these large phenotypic studies with whole genome sequencing and transcriptome analysis has the potential to power large-scale genotype–phenotype studies of invasion phenotypes, just as have been carried out for drug-resistant phenotypes [42–48].

Such large-scale studies of invasion phenotypes in field samples using standardized methods also have the potential to play a very significant role in vaccine development. Field testing of vaccine candidates is an expensive and logistically difficult challenge. Phase II studies of blood-stage vaccines, for example, have as a primary focus the question of whether a single vaccine can protect against the wide range of genotypes and phenotypes found in field *P. falciparum* strains, rather than the few laboratory strains that the vaccine would have been tested on during preclinical and Phase I studies. A network of laboratories, all using standardized protocols to study variation in invasion phenotypes, could systematically support vaccine candidate assessment at the preclinical stage. Invasion phenotyping using the standardized assay could be used to map the extent of invasion variability in a given

region, while parallel studies using clinical trial sera and/or standardized reagents such as monoclonal antibodies against vaccine candidates would test the reliance of each strain on a given target. Performing such studies across the network, testing thousands of parasite isolates from different countries and endemicities in a coordinated manner, would fill a critical gap in the invasion vaccine development pipeline for a relatively modest investment of resources (certainly more modest than a Phase IIb vaccine trial). Such an approach would provide clear data for downselection and elimination of targets, a step that has often been missing in blood-stage vaccine development.

Concluding Remarks

The recent changes in DNA sequencing technology have radically changed the scale of studies of parasite genetic variation, with analyzing the genomes of hundreds if not thousands of parasites becoming the goal for many groups, as well as for large multigroup collaborations such as MalariaGen (www.malariagen.net). To date, the only studies of parasite phenotypic variation to approach this scale have focused on drug resistance. Our goal is to extend these large-scale phenotypic studies to erythrocyte invasion, to drive vaccine development as well as biological understanding. As outlined in this opinion article, the WAMIN network has been established to develop a robust and reproducible protocol for phenotyping erythrocyte invasion in the field, and to build the technical and skills capacity to apply that protocol across multiple sites in West Africa. As a next step, we plan to systematically test the areas identified in the Outstanding Questions box where more research is required before a final SOP can be developed. This SOP will then be rigorously tested at a second WAMIN workshop, which will also establish a harmonized method for data analysis. We aim to subsequently apply this approach to a large-scale study of a few thousand isolates across West Africa. The goal of this large-scale study will be to phenotype natural isolates with enough power to conduct GWAS and establish the reservoir of invasion diversity with high confidence, as well as to use standardized reagents to systematically test invasion vaccine targets to power critical go/no-go decisions. Such a coordinated systematic approach may also lead the way for studies of other aspects of parasite biology on a large scale in the real world

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Box 1.**Recommendations for Standardization**

The following recommendations represent areas of agreement that must be standardized for data to be comparable across geographically distinct sites.

Storage of Enzymes and Reagents:

All enzymes and reagents must be stored at the appropriate temperature at all times. Expiration dates must be strictly enforced. It is recommended that thermometers with temperature tracking capabilities should be installed, and if temperatures fluctuate within a given margin of error, reagents must be discarded or tested using known standards.

Minimize Batch Variation:

Batch variation, especially for enzymes, antibodies, and other inhibitors is a major potential source of error, even within the same laboratory using the same protocol. Great efforts should be made to limit the effects of batch variation, and all laboratories in the network should share the same batch of reagents wherever possible. Batches should be tested and validated, and ideally would be purchased for use by all laboratories, although this requires some centralization of resources for supply, shipping, and technical management.

Parasite and Erythrocyte Preparation and Storage:

Variation between erythrocyte acceptor cells may affect the results of the assays, since blood group variation can influence invasion efficiency. However, a single source of erythrocytes is not practical across multiple sites, especially given that laboratory storage of acceptor cells can also affect the data. A detailed series of tests of the impact of age and common blood groups on invasion is needed before setting a final recommendation. The current recommendation is for all sites to use only cells that are no more than 7 days old in 4 °C storage.

Avoiding Antimalarial Exposure:

Before antimalarial exposure can dramatically affect parasite viability and reinvasion, a number of recommendations are put in place to avoid the confounding of antimalarial exposure: (i) patients who self-report as having taken antimalarials are not eligible to participate in the study (and this exclusion criteria should be enforced across all sites); (ii) infected erythrocytes are washed three times with unsupplemented RPMI to remove any unreported antimalarials; (iii) if parasite multiplication rates are less than 1, the assay is not included in analysis because of suboptimal reinvasion.

Stage of the Life Cycle to Set Up the Invasion Assay:

While some laboratories set up the invasion assay using schizonts, others use ring stages. There are advantages in either approach: for collaborative research projects, we propose that all participating laboratories should use the ring stage. This will help to generate data that will be comparable between sites.

Inclusion of Control Strains and Standards:

To allow for internal control of assay success, and to help validate whether well-stored and controlled reagents are continuing to work as they should, we recommend the inclusion of cloned, identical control strains for each acceptor cell treatment. The network will clone control strains (3D7, Dd2, HB3) from a single whole-genome sequenced source, and clones will be expanded enough to provide to each participating research center. Thus, every control strain used in an assay will be from a replicate vial of the same stock, similar to what is the norm for standardized drug assays. For antibody inhibition assays, commercially available internal standards (such as anti-BSG, clone MEM-M6/6) at two different concentrations will be included that should give the same level of inhibition for every assay, for a given strain. This will assure internal quality control of the assay. All invasion assays should include at least one standard strain, and all inhibition assays include at least one standard inhibition reagent.

Dual Dye Combinations:

Different combinations of dyes are used to label and quantitate cells for both acceptor cells and parasites for the invasion assay. While the choice often depends on the model of flow cytometry being used, standardized dyes would greatly increase comparability. For dual-color flow-cytometry applications, we recommend the use of DDAO for labeling acceptor cells and SYBR Green for staining infected erythrocytes (Figure 3).

Trends

Merozoite invasion is a promising vaccine target.

Studies of invasion in the field will be essential to advance vaccine development.

A new network, WAMIN, has been formed to optimize and standardize invasion assays.

WAMIN will prioritize vaccine targets by phenotyping thousands of field parasites.

Outstanding Questions

While some recommendations for standardization can be made now, others need investigation. The following areas include sources of both technical and biological variation that will require systematic testing by the network before formal recommendation and inclusion in the SOP.

How does cryopreservation affect parasite phenotypes? Fresh isolates are as close as possible to the *in vivo* phenotype of the parasites, which makes this approach very attractive. However, this has to be balanced by the requirement for well-equipped laboratories with sterile tissue culture hoods, incubators, parasite gas, microscopes, and a flow cytometer to perform invasion assays close to where sample collection occurs. This would exclude remote areas, and as a result potentially very significant phenotypes may be missed. The possible impact of cryopreservation on parasite phenotypes needs to be systematically tested.

How does initial parasitemia affect the result of invasion assays? Some laboratories adjust the initial parasitemia for setting up the invasion assays, while other laboratories set up the assays at different initial parasitemias, based on the *in vivo* parasitemia. Culture conditions and initial parasitemia for use in assays need to be optimized and clearly defined.

How can potential donor cell confounders best be eliminated? Similar to the challenge of acceptor cell variation, donor cells can also pose an equally relevant source of variation. Donor cells will come from thousands of different patients and may be more or less susceptible to invasion than the acceptor cells, depending on genetic background differences. One option to address this would be to enzymatically treat donor cells with all three enzymes to remove all receptors and render them nonpermissive to invasion (Figure 3). The impact of donor variation and pretreatment of donor cells needs to be tested.

How should antibodies be prepared and purified? As with invasion assays, each laboratory has a different method of antibody purification (size exclusion columns, IgG purification, protein-specific antibody purification by fast protein liquid chromatography), inclusion/exclusion of complement, and a different antibody concentration to test for inhibition. As one of the goals of the WAMIN network will be assessing the inhibitory potential of antibodies to leading vaccine candidate antigens, the method of antibody preparation and concentration represents a key area for standardization.

What is the best way to evaluate invasion ligand expression? Since invasion ligand expression levels can influence invasion pathway utilization and inhibition, having a rapid, quantitative, and standardized method for assessing ligand expression of each strain tested will be an important development. Methods such as RNA profiling by RT-qPCR or RNAseq and/or protein profiling by quantitative Western with a panel of antibodies will be explored as potential additions for standardization.

Figure 1. Erythrocyte Invasion is a Complex Process Involving Multiple Steps.

(A) Mature merozoites are released from the bursting schizont, and disperse among surrounding red blood cells. Merozoites initially contact, probably at random, uninfected erythrocytes and loosely adhere on their cell surface. (B) Primary merozoite contact is associated with an obvious warping of erythrocyte cell membrane during which the merozoite reorients itself to place its apical end in direct apposition with the erythrocyte cell membrane [49]. Merozoite reorientation is followed by the establishment of additional protein–protein contacts between the two juxtaposed cell membranes, probably committing the merozoite to invasion [50–52]. (C) These contacts are followed by the formation of a so-called tight or moving junction, which is transformed into a migrating ring (the moving junction) that moves towards the basal end of merozoite, moving the merozoite into the erythrocyte [14,53]. The function of ligands at each step is not completely well established, although the erythrocyte binding-like (EBL) and reticulocyte binding protein homolog (RH) ligands are believed to bind to specific erythrocyte receptors at some point between the initial merozoite attachment and the formation of the tight junction [14,52]. Merozoite thrombospondin-related anonymous protein (MTRAP) is thought to bridge the gap between parasite actin–myosin motor and host erythrocyte plasma membrane during the moving junction step [54,55], which is mediated by the apical membrane antigen (AMA1)–rhoptry neck protein (RON2/4/5) complex.

(A)

(B)

Figure 2. Previous Studies Using Enzymatic Treatment of Erythrocytes to Examine Invasion Phenotypes of *Plasmodium falciparum* Clinical Isolates.

(A) Global distribution of the ten different endemic countries where erythrocyte invasion phenotypes have been determined. Phenotypes of 536 clinical *P. falciparum* isoaltes have been reported in 13 different research papers; number of isolates studied from each country are shown in parentheses [12,17,23,32–37,31,38–40]. A large majority of the isolates (437, 81.5% of the total) were from patients in West Africa. (B) Summary of the seven studies [17,23,32,34,37,38,40] that have investigated erythrocyte invasion phenotypes of West African clinical *P. falciparum* isolates. The data highlight the differences in numbers of isolates tested, and concentrations (per ml) of neuraminidase (Neu), chymotrypsin (Chy), and trypsin (Try) used for erythrocyte treatment. Senegal 1 [37]; Senegal 2 [32]; Senegal 3 [23]; Gambia 1 [34]; Gambia 2 [38]; Ghana 1 [17]; Guinea, Senegal 4, Ghana 2 [40].

Figure 3. Example of a Merozoite Invasion Assay Protocol.

Erythrocytes to be invaded (acceptor cells, blue) are fluorescently labeled with DDAO-SE and treated with combinations of the standard enzymes used for merozoite invasion phenotyping – trypsin (Try), chymotrypsin (Chy), and neuraminidase (Neu) – or left untreated [56]. Acceptor cells are then incubated with parasitized erythrocytes (donor cells, red) that have been rendered nonpermissive for invasion by enzymatic treatment. If desired, antibodies against specific receptors or ligands may be included to determine their inhibitory activities. Parasites are allowed to invade over 48 h in culture, then erythrocytes are stained with SYBR Green I to label parasite DNA and analyzed by flow cytometry. Acceptor erythrocytes that have been invaded are identified as DDAO-SE-positive and SYBR Green I-positive cells. Even with enzyme treatment, there can be low levels of background invasion

into donor cells or, alternatively, nonviable schizonts or gametocytes can be present; thus, distinguishing between parasites in donor and acceptor cell populations is crucial to measuring true reinvasion efficiency. Comparing invasion in untreated and enzyme-treated cells reveals the invasion phenotype of the parasites in the acceptor cells.

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