Published in final edited form as:

Exp Parasitol. 2010 October ; 126(2): 198–202. doi:10.1016/j.exppara.2010.04.022.

Cloning of *Plasmodium falciparum* by single-cell sorting

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Abstract

Malaria parasite cloning is traditionally carried out mainly by using the limiting dilution method, which is laborious, imprecise, and unable to distinguish multiply-infected RBCs. In this study, we used a parasite engineered to express green fluorescent protein (GFP) to evaluate a single-cell sorting method for rapidly cloning *Plasmodium falciparum*. By dividing a two dimensional scattergram from a cell sorter into 17 gates, we determined the parameters for isolating singly-infected erythrocytes and sorted them into individual cultures. Pre-gating of the engineered parasites for GFP allowed the isolation of almost 100% GFP-positive clones. Compared with the limiting dilution method, the number of parasite clones obtained by single-cell sorting was much higher. Molecular analyses showed that parasite isolates obtained by single-cell sorting were highly homogenous. This highly efficient single-cell sorting method should prove very useful for cloning both *P. falciparum* laboratory populations from genetic manipulation experiments and clinical samples.

Index Descriptors

Plasmodium falciparum; transfection; parasite cloning; flow cytometry, cell sorting

1. Introduction

Currently, malaria remains as one of the most serious public health problem in the world, causing a death toll of almost a million per year, mostly in sub-Saharan Africa (Snow et al., 2005). Both basic and translational researches aimed at a better understanding of the molecular, biochemical and immunological aspects of the malaria parasites are needed to develop and implement novel control measures such as vaccines and drugs. Because natural malaria parasite populations frequently contain genetically heterogeneous strains, and patients in hyperendemic regions are often infected by mixed parasite strains (Zhong et al., 2007), many in-depth studies of the parasites in the laboratory require cloned parasites from clinical samples. For example, in vitro monitoring of drug resistance needs to be performed on cloned parasites, since assay results derived from mixed parasite strains are not accurate (Liu et al., 2008). In addition, functional genomic studies by genetic manipulation of the parasites also require analysis of single parasite clones.

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Limiting dilution is a commonly used method for cloning cells, including the malaria parasite *Plasmodium falciparum* (Francois et al., 1994, Rosario, 1981). To clone the malaria parasites, infected red blood cells (RBCs) are diluted to a desired parasitemia so that infected RBCs can be divided into single culture units. However, this method has several intrinsic limitations. It is a tedious procedure, requiring accurate quantification of the parasitemia. Besides, it is unable to distinguish multiply infected erythrocytes, which are common under stationary culture conditions, necessitating subsequent confirmation analysis of the resultant clones. While traditional parasitemia determination by microscopic counting of blood smears is tedious and imprecise, flow cytometry-based methods provide significant improvement in terms of time and accuracy. Flow cytometry has been used in malaria diagnosis (Saito-Ito et al., 2001, Whaun et al., 1983), quantifying parasitemia (Barkan et al., 2000, Bhakdi et al., 2007, Kadjoian et al., 1992, Li et al., 2007), determining parasite developmental stages (Liu et al., 2005, Pavithra et al., 2004), and cell viability (Russo et al., 2009). Furthermore, technical advance in cell sorting allows high-speed, accurate sorting and collection of individual cells based on user-defined flow cytometry parameters (Ibrahim and van den Engh, 2007). As such, the cell sorter has become a powerful technique and starting point for downstream studies. In malaria research, cell sorting has been used to isolate infected liver cells (Tarun et al., 2006, 2008), GFP-labeled rodent parasites (Janse et al., 2006), and sexually differentiated gametocytes (Khan et al., 2005).

Analysis of single cells has been used in both basic and applied scientific fields. Cell sorting can be based on fluorescent labels and light scattering properties of the cells. Fluorescent labels, such as with specific antibodies, are useful for isolating specific cell types. Labels can also be used to amplify certain cell features, allowing easier identification and isolation of specific cell populations. However, certain labels such as those for labeling DNA may damage the cells, and thereby are not suitable when proper cell physiology needs to be retained for subsequent studies such as measuring cell growth rate. Accordingly, sorting cells by using only the light-scattering properties can differentiate cell populations by their size, shape and content, thus avoiding cell damages (Ohnuma et al., 2006).

In this study, we seek to employ the cell sorter to isolate single malaria parasite infected RBCs to individual culture units without cell staining. This method is much more efficient and accurate than the traditional limiting dilution method for parasite cloning. It can be used for cloning *P. falciparum* and isolating singly-infected erythrocytes.

2. Materials and Methods

2.1. Generation of fluorescently labeled parasites

To develop the method of parasite cloning by single-cell sorting using parasites from transfection studies, we generated a construct, PfGCN5-GFP, in order to tag the endogenous *PfGCN5* (*PF08_0034*) with green fluorescent protein (GFP) at the C-terminal end through a single crossover event. A 1013 bp *PfGCN5* fragment was amplified with primers GF (5'GAAGATCTGCTGTTACCTCAACTGAGC 3') and GR (5'CACCTAGGTGCTGTATCAGTTATAGCTTC 3') and cloned at the *Bgl*II and *Avr*II sites in pBlue-GFP (Fan et al., 2009). Then, the *PfGCN5-GFP* fragment plus pDT3' was released by digestion with *Bgl*II and *Not*I and cloned into pHD22Y to generate the transfection plasmid pHD22Y/PfGCN5-GFP. *P. falciparum* clone 3D7 was maintained using a standard procedure (Trager and Jensen, 1976) and synchronized by sorbitol treatment (Lambros and Vanderberg, 1979). For transfection, ~100 µg of plasmid DNA were electroporated into 3–4% of ring stage parasites (Fidock and Wellem, 1997). Two days later, parasites were selected with WR99210 at 2.5 nM for ~3 weeks with weekly replenishment of fresh RBCs until resistant parasites were seen. Parasites containing

integrated forms of the constructs were enriched by cycling the parasites three times with or without the selection drug.

2.2. Flow cytometry and cell sorting

To determine the criterion for single cell sorting, 3D7 parasites were cultured and synchronized by sorbitol treatment. Parasites at the trophozoite stage were purified on 65% Percoll, washed twice with RPMI1640, and injected into Cytopeia Influx high speed sorter (BD Biosciences) under sterile conditions at room temperature. All measurements were made using argon laser at 488 nm. The cell sorting conditions were as follows: flow chip diameter, 100 μ m; sheath solution, PBS; sheath pressure, 52 PSI; crystal frequency, 85 KH; crystal drive, 12.2% delay, 44 drops; sort mode, purity-yield; coincident abort, on. Forward angle light scatter (FSC) and side angle light scatter (SSC) were set at 24 and 44.25% photomultiplier tubes (PMT) power, respectively. The cell particles were assessed and plotted in a two dimensional scattergram of FSC and SSC in linear display in less than 60 s. In order to determine the content of particles (RBCs, RBCs with one, two or more parasites, aggregated RBC, or cell debris) in the plotted area, FSC and SSC were divided into several sections and 17 fractions (gates), and cells in different areas of the forward and side scatter were sorted into individual tubes (10^6 cell per fraction) (Fig. 1). These sorted cells were used to make thin smear, stained with Giemsa, and examined by microscopy.

Fractions 12 and 13 that had most population with one parasite per RBC were used for cloning by single-cell sorting. Parasites transfected with pHD22Y/PfGCN5-GFP were cloned by single cell sorting to determine the efficiency and accuracy of this parasite cloning procedure. Ninety-six well plates were preloaded with 200 μ l per well of RBCs at 1% hematocrit in RPMI 1640 medium containing 10% human sera, and the programmable micro-titer well tray configurations set at depositing a single cell per well. The plate was then incubated in a candle jar at 37°C, and medium was changed on day 5 and 10. Slides from each well were made and examined on day 12, and parasite-positive cultures were expanded first to 24 well plates and subsequently to 25 cm² flasks. The selection drug WR99210 at 2.5 nM was added to the culture at about four weeks after sorting. In parallel, parasite cloning was performed using the limiting dilution procedure (Rosario, 1981).

Since the integration of transfection plasmid could occur at several loci in the parasite genome, but only plasmid correctly integrated at the *PfGCN5* locus will result in GFP expression, we first sorted out the GFP-positive parasites by gating the parasites with the GFP filter (531 –40 at 42.10% PMT) to pre-select the GFP-positive parasites for cloning. The GFP-positive cells were then subjected to selection by FSC and SSC as described above, and the fractions of parasite population harboring one parasite per RBC were used for cloning by single-cell sorting.

3. Confirmation of single clone by drug selection, polymerase chain reaction (PCR), Southern blot and GFP imaging

To verify that parasites from each well of the 96-well plate were derived from a single cell, we first applied WR99210 at 2.5 nM to the culture to determine if the culture was drug-resistant or -sensitive. We then determined whether the plasmid was properly integrated at the *PfGCN5* locus by PCR and genomic Southern blot. Three pairs of primers were designed to detect the correct integration event at *PfGCN5* locus by PCR. Primers F1 (5'-GGTGGAGTATGTTTCAGACC-3') and R1 (5'-CATTTTCCTTTACGAAAAAGAG-3'), located upstream and downstream of *PfGCN5* C-terminus respectively, would amplify a 1095 bp fragment if there was no integration. If the plasmid was integrated at the *PfGCN5* locus, there would be no amplification product under the current PCR conditions since the integration event would insert a more than 7 kb

fragment between the two primers (Fig. 2A). Primers F1 and R2 (5'-GGACAACCTCCAGTGG-3') in the *GFP* gene were used for confirming the integration event (1258 bp). Primers GF and M13R were chosen to detect if parasites contained the plasmid. Genomic Southern blot was performed to further confirm the integration event. Parasite DNA was extracted from cultured parasites using a proteinase K digestion and phenol: chloroform extraction procedure (Cui et al., 2002). Three μ g of genomic DNA from each clone and 6 μ g from wild-type 3D7 were digested with *Bam*HI and *Spe*I and separated on a 0.8% agarose gel. DNA was transferred to a nylon membrane (Osmonics, MA), denatured, and UV-crosslinked. About 25 ng of PCR product of the *GFP* gene were labeled with (α -³²P)dATP using Prime-A-Gene system (Promega, WI). The membrane was prehybridized in 7% SDS, 0.5 M sodium phosphate monobasic (NaH_2PO_4 , pH 7.2), 2% dextran sulfate for 3 h at 65°C and hybridized with the denatured probe for 12 h at 65°C (Kyes et al., 2000). Washes were done twice at 60°C for 20 min with $2 \times \text{SSC}/0.5\%$ SDS and once for 50 min with $1 \times \text{SSC}/0.1\%$ SDS. The membrane was exposed to a Kodak X-ray film at -80°C for autoradiography. All parasite clones were observed under a fluorescence microscope to confirm GFP labeling.

3. Results

3.1. Determination of parameters for effective sorting of singly infected erythrocytes

Synchronized 3D7 parasites at the trophozoite stage were purified on 65% Percoll, which contained more than 95% parasitized RBCs. Depending on the culture conditions, we found that more than 10% of the parasitized RBCs contained more than one parasite, and higher parasitemias tended to result in higher percentages of multiply-infected RBCs. This result was consistent with the finding by Allen and Kirk (Allen and Kirk, 2009). In order to sort out singly-infected RBCs, we divided the area of forward and side scatters into 5 to 6 sections, and a total of 17 gates were set up to sort respective fractions (Fig. 1). Examination of different fractions of the sorted parasites revealed that fractions 12 and 13 contained nearly 100% singly-infected RBCs (Table 1). Fractions from higher forward and side scatter area had higher percentages of RBCs harboring more than one parasite, while the lower areas had more uninfected RBCs and cell debris (Table 1, Fig. 1).

3.2. Single-cell sorting for parasite cloning

We used fractions 12 and 13 for single-cell sorting of infected RBCs, and sorted cells were individually deposited in the wells of a 96-well culture plate. From the sorting experiment with parasites transfected with pHD22Y/PfGCN5-GFP, parasites were detected in ~90% of the wells (86/96 and 87/96 wells from two 96-well plates) after approximately two weeks of culture. About 95% of these parasites (82/86 and 83/87) were successfully expanded into 25 cm² flasks and showed sustained growth for over a week under 2.5 nM of WR99210, suggesting that most of parasite clones had plasmid integration into the genome. When analyzed by PCR, 97% of the WR99210-resistant clones (80/82 and 80/83) produced PCR product of the predicted size with primer F1 and R2, but not with primers F1 and R1, suggesting of plasmid integration at the *PfGCN5* locus (Fig. 2B, left panel). PCR from 2/82 and 3/83 of the clones showed the opposite, suggesting of plasmid integration into other regions of the genome (Fig. 2B, middle panel). Southern blot confirmed the integration events (Fig. 2C). In comparison, cloning by limiting dilution with the same parasite sample only resulted in ~30% wells (27/96 and 30/96) with parasite growth, and 92% (25/27 and 27/30) of them were resistant to WR99210. PCR detected that 90% of the drug-resistant parasites (22/25 and 24/27) had GFP integration at the *PfGCN5* locus. For the remaining six wells, two from each plate (2/25 and 2/27) had plasmid integrated elsewhere, and one clone from each plate produced PCR products with primers F1 $\times$ R1 and F1 $\times$ R2, suggesting that these two wells contained a mixture of parasites with the plasmids integrated at the *PfGCN5*

locus and at another site of the genome (Fig. 2B, right panel). Southern blot could detect these two integration events in these clones (Fig. 2C). All clones with predicted GFP-tagging at the PfGCN5 locus were GFP-positive, whereas drug-sensitive clones and resistant clones with plasmid integrated elsewhere showed no GFP signal. The clones predicted to contain mixed parasite clones had both GFP-positive and negative parasites.

We tried to enrich GFP-positive parasites before performing single-cell sorting with the transfected parasites. As shown in Figure 3A, flow cytometry analysis detected that 14% of parasites produced a much stronger GFP signal. After pre-selection of this fraction, these GFP positive cells were subjected to single-cell sorting. From this experiment, parasites developed in 85 and 88 wells of two 96-well plates. PCR and Southern blot confirmed that all parasites had correct integration of the plasmid at the PfGCN5 locus (data not shown). All these parasites showed GFP under fluorescence microscope (Fig. 3B).

4. Discussion

Malaria parasite cloning is an important method for basic and applied research. The commonly used method of limiting dilution is time-consuming and labor-intensive, presenting a challenge for large-scale cloning of parasites from clinical samples. Many previous researches have applied flow cytometry to measure parasitemia and determine the relative abundance of parasites at different stages, but the uses of DNA or RNA dyes in these procedures make it unsuitable for parasite cloning. In this study, we took advantages of the high efficiency and accuracy of the cell sorter for parasite cloning, and determined the parameters for sorting out singly-infected RBCs without incorporation of dyes.

During the asexual intraerythrocytic developmental cycle, the malaria parasite undergoes dramatic changes in size and shape. Although flow cytometric parameters can be determined to distinguish different parasite stages and to sort singly-infected cells from an asynchronous culture, we found that synchronization of the culture and purification of the trophozoite stages using Percoll greatly simplify the single-cell sorting procedure. By defining the FSC and SSC parameters, we could identify parasite populations with >95% of infected RBCs containing only one parasite. Using our defined conditions, single-cell sorting of parasite populations from a middle scatter area resulted in 80% of culture units containing viable parasites. This is much higher than the cloning efficiency from the limiting dilution procedure (30%). Since stationary parasite culture often contain significant proportions of RBCs containing more than one parasite, cultures that are used for single-cell sorting may be maintained under shaking conditions in order to reduce the proportion of multiply infected RBCs (Allen and Kirk, 2009). We also found that this single-cell sorting method can be coupled to experiments for generating fluorescently labeled parasites. Altogether, this work demonstrated that single-cell sorting technique using a cell sorter can be a highly efficient and accurate method of parasite cloning. In addition, this method can be applied for isolating individual cells for single-cell level analysis (Chen et al., 1998). Furthermore, cloned parasites from field isolates can be verified using molecular markers such as polymorphic antigens and microsatellites. These parasite clones are very useful for more accurate studies of population genetics, in vitro drug resistance, and invasion phenotypes, etc.

Abbreviations

RBCs	red blood cells
GFP	green fluorescent protein
FSC	forward angle light scatter

SSC side angle light scatter

Acknowledgments

This work was partially supported by NIH (R01 AI064553).

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Fig. 1. Scattergram of infected RBCs and determination of FSC and SSC parameters for isolating singly-infected RBCs

Purified trophozoites were subjected to cell sorting, and the FSC and SSC scattergram was shown in the left panel. From this scatter pattern, 17 gates were set to cover the plotted area (right panel). 10^6 particles from each gate were sorted into individual tubes, made into thin smears, and analyzed by Giemsa staining. Gates 12 and 13 contained mostly singly-infected RBCs.

Fig. 2. Tagging of the C-terminus of *PfGCN5* with GFP and characterization of clones
(A) Predicted GFP integration from a single-crossover event. Restriction enzyme *Bam*HI sites (B) and the expected sizes of DNA fragments after *Bam*HI digestion are shown. Top: the *PfGCN5* locus on chromosome 11. Solid lines indicate introns or intergenic regions, filled boxes are exons, and hatched boxes are the C-terminal end for homologous recombination. Middle: The transfection plasmid pHD22Y/GCN5-GFP showing the *PfGCN5* genomic fragment fusion with GFP, pDT 3', and the drug selection cassette *hDHFR*. Bottom: The predicted single-crossover event at the *PfGCN5* locus showing the integration of one copy of the plasmid. The primers F1 × R2, GF × M13R and F1 × R1 were designed for identifying this integration event, confirmation of the presence of the transfected plasmid, and detecting the intact *PfGCN5* locus, respectively. **(B)** Confirmation of chromosomal integration and clones by PCR. Genomic DNA of wild-type 3D7 and clones were amplified with primers F1 × R2, F1 × R1 and GF × M13R, and the sizes of the PCR products are indicated. All clones (except the wild type 3D7) were positive for GF × M13R, indicating the presence of the transfection plasmid in the parasites. Four clones (1, 2, 3 and 4) showed PCR amplification with F1 × R2, but not F1 × R1, suggesting of GFP integration at the *PfGCN5* locus and no contamination with parasites with other integration events. Four other clones (a, b, c and d) showed the opposite - negative with F1 × R2, but positive with F1 × R1, suggesting that the *PfGCN5* was intact and plasmid was integrated elsewhere. Two wells (A and B) derived from limited dilution method showed PCR products for all three pairs of primers, suggesting that these parasites were not clonal and contained a mixture of parasites with GFP integrated at the *PfGCN5* locus and elsewhere in the genome **(C)** Verification of the predicted integration events and resulting clones by

Southern blot. Genomic DNA from wild type 3D7, four clones with integration at the *PfGCN5* locus (1, 2, 3 and 4), one clone with plasmid integrated at another locus of the genome (a), 100 ng of the plasmid (P), and two isolates containing mixed clones (A and B) was digested with *Bam*H1, separated in a 0.6% agarose gel, and hybridized to ³²P-labeled GFP PCR product. Integration at the *PfGCN5* locus only showed a 13.7 Kb band, whereas integration of the plasmid at another locus had a 10.5 kb band. Two bands were observed in isolates A and B, suggesting of mixed clones.

Fig. 3. Pre-gating of GFP-expressing parasites before single-cell sorting

(A) GFP expressing trophozoites were selected by setting a gate (M1). Parasites were scanned with the GFP filter to measure the GFP fluorescence intensity of each parasite. Parasites with GFP signal intensity falling within the M1 gate were selected for single-cell sorting. (B) GFP images of single clones from single-cell cloning were captured under a fluorescence microscope.

Table 1
The composition of parasites sorted using the 17 gates derived from the scattergram

Sorted materials of purified trophozoites from each gate were used to make Giemsa-stained thin smears, and the percentages of uninfected RBCs, RBCs infected with one, two, three or more parasites, and cell debris were determined.

Gate	Parasite numbers per RBC				RBCs	Cell debris
	1	2	3	>3		
1	20	28	30	21	0	1
2	25	17	23	28	2	5
3	30	6	0	0	25	39
4	73	23	3	1	0	0
5	75	19	5	1	0	0
6	69	17	9	4	0	1
7	83	17	0	0	0	0
8	89	11	0	0	0	0
9	86	13	1	0	0	0
10	84	14	1	0	0	1
11	94	6	0	0	0	0
12	99	1	0	0	0	0
13	98	2	0	0	0	0
14	95	4	1	0	0	0
15	93	2	0	0	1	4
16	93	3	0	0	1	3
17	92	3	1	0	1	3