Isolation of Plasmodium falciparum by flow-cytometry: implications for single-trophozoite genotyping and parasite DNA purification for whole-genome high-throughput sequencing of archival samples.

Anne Boissière, Céline Arnathau, Christophe Duperray, Laurence Berry, Laurence Lachaud, François Renaud, Durand Patrick, Franck Prugnolle

To cite this version:
Anne Boissière, Céline Arnathau, Christophe Duperray, Laurence Berry, Laurence Lachaud, et al.. Isolation of Plasmodium falciparum by flow-cytometry: implications for single-trophozoite genotyping and parasite DNA purification for whole-genome high-throughput sequencing of archival samples.. Malaria Journal, BioMed Central, 2012, 11, pp.163. 10.1186/1475-2875-11-163. inserm-00802619

HAL Id: inserm-00802619
https://www.hal.inserm.fr/inserm-00802619
Submitted on 20 Mar 2013

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Isolation of *Plasmodium falciparum* by flow-cytometry: implications for single-trophozoite genotyping and parasite DNA purification for whole-genome high-throughput sequencing of archival samples

Anne Boissière1*, Céline Arnathau1, Christophe Duperray2, Laurence Berry3, Laurence Lachaud1, François Renaud1, Patrick Durand1 and Franck Prugnolle1*

**Abstract**

**Background:** Flow cytometry and cell sorting are powerful tools enabling the selection of particular cell types within heterogeneous cell mixtures. These techniques, combined with whole genome amplification that non-specifically amplify small amounts of starting DNA, offer exciting new opportunities for the study of malaria genetics. Among them, two are tested in this paper: (1) single cell genotyping and (2) parasite DNA purification for subsequent whole genome sequencing using shotgun technologies.

**Methods:** The method described allows isolation of *Plasmodium falciparum* trophozoites, genotyping and whole genome sequencing from the blood of infected patients. For trophozoite isolation, parasite and host nuclei are stained using propidium iodide (PI) followed by flow cytometry and cell sorting to separate trophozoites from host cells. Before genotyping or sequencing, whole genome amplification is used to increase the amount of DNA within sorted samples. The method has been specifically designed to deal with frozen blood samples.

**Results and conclusion:** The results demonstrate that single trophozoite genotyping is possible and that cell sorting can be successfully applied to reduce the contaminating host DNA for subsequent whole genome sequencing of parasites extracted from infected blood samples.

**Keywords:** Plasmodium, FACS, Cytometry, Genotyping, Whole genome sequencing

**Background**

Flow cytometry and cell sorting are powerful technologies that allow the physical separation of a cell, or a particle of interest, from a heterogeneous cell mixture. These techniques, combined with specific technologies in genetics such as whole genome amplification, provide new opportunities for malaria genetic studies. Among them two are explored in this article: (i) single trophozoite genotyping and (ii) parasite DNA purification for subsequent next generation shotgun sequencing.

**Single cell genotyping**

Hosts are often infected with more than one genotype of the same pathogen. Multiclonal infections arise from infections with a genetically diverse inoculum or from re-infection before an existing infection is cleared [1]. Ecological interactions between these lineages within hosts can influence the parasite's life history traits (virulence, transmission) and can thus have important consequences for their epidemiology and evolution [2,3].

Despite their ecological and evolutionary importance, mixed infections are frequently overlooked in natural conditions. This is true for malaria parasites, where most population genetic studies are performed using total DNA extracted from blood samples of infected patients.
In areas of low transmission, most patients are infected with only one haploid parasite (one haplotype) whereas in areas of high transmission, a large proportion of patients carry multiple haplotypes [4]. Although both single and mixed-infections can be used to globally estimate allele frequencies at each locus [5], it remains difficult to assess the combinations of alleles at different loci. This information can yet be fundamental for understanding the population dynamics, evolution and biology of the parasite. Several methods have been proposed to circumvent this problem. One is to adapt the patient sample to culture and then dilute it to create monoclonal cultures [6]. However it is only possible to apply this method to very fresh, or cryopreserved, samples and it is very labour intensive. In addition this method may select for culturable variants. A second possibility is to use statistical methods to predict putative haplotypes. Several methods exist but, practically, they are difficult to apply to mixed samples containing more than two malaria strains [7,8]. As a consequence, when the allele combinations at different loci are the point of interest, mixed infections are often discarded to avoid analytical problems intrinsic to the handling of mixed genotypes.

One possibility to overcome these problems associated with multiple infections is to work directly at the scale of one parasite (one genome) and to genotype individual parasites contained in a patient’s blood. Such methods, unfathomable a couple of years ago are now possible thanks to the development and improvement of different tools. These include: i) efficient means to isolate and sort single cells, such as fluorescence-activated cell sorting [9], or laser capture micro-dissection; and ii) whole genome amplification (WGA) tools which allow the generation of a high quantity of genomic DNA from a very small initial quantity. Until now, single-cell sorting and genotyping have not been tested with malaria parasites.

*Plasmodium falciparum* DNA enrichment and whole genome sequencing

Another area of interest of flow cytometry and cell sorting for the study of malaria genetics is the separation of parasite cells from those of the host for subsequent whole genome sequencing of the parasite.

One major problem to sequencing malaria infected blood samples using shotgun technologies is the presence of “contaminating” host DNA [10]. Because of the large size discrepancy between the human and *Plasmodium* genome, the presence of even a small number of host cells compared to parasite cells may preclude to obtain good sequence coverage of the parasite’s genome at a reasonable cost. Methods that enrich DNA samples with parasite DNA, or that remove host nucleated cells from samples, are imperative for efficient sequencing of the parasite genome [11,12].

Several methods have been proposed to purify the parasite DNA before shotgun sequencing. Sustained *in vitro* culture of the parasite in a human DNA free medium is one possibility. This method nevertheless demands a lot of time and expertise and is currently limited to species that can be adapted in culture. Another possibility is to selectively remove the leucocytes from the patient’s blood. Several methods have been proposed with various degrees of efficiency but they all require samples for which cytoplasmic membrane integrity is preserved (i.e. very fresh samples or cryopreserved samples in medium that maintains membrane integrity) [10]. Finally, a method based on hybrid selection of the targeted genome has been proposed. Its principle is to enrich the parasite DNA from a DNA mixture by specifically hybridizing it on special baits [11]. This technique can be applied to any DNA extract obtained from fresh or frozen archived samples at a modest cost. One limitation of this method however is that it can only be applied to pathogens for which complete genome sequences are known, or those of very closely related species. In addition, it is likely that regions of the genome displaying high levels of polymorphism (such as regions coding for antigenic proteins) could be underrepresented in the final sequence due to low affinity with baits during hybridization.

Again flow cytometry and cell sorting can provide a good alternative to these methods for several reasons: (i) they can be applied to any *Plasmodium* species infecting any mammal host, as long as it is possible to distinguish the parasite populations from the host nucleated blood cell population, (ii) they can be applied to any kind of blood samples, fresh or frozen, as long as enough host and trophozoite nuclei (which have very different sizes) remain intact for sorting and separation.

In this paper, flow cytometry and cell sorting technologies were tested for these two applications. The aim was to isolate trophozoites using fluorescence-activated cell sorting from the blood of infected patients, and genotype or sequence them, following whole genome amplification. The present protocol was developed from previously published protocols and was specifically designed to work on archived infected blood samples that have been conserved frozen.

**Methods**

**General method of isolation and applications**

The detailed protocol outlines trophozoite cell preparation from archived frozen samples (conserved at minus 20°C), characterization and cell isolation. It is followed by whole-genome amplification that provides a template for single-cell microsatellite genotyping and multiple-cell whole-genome sequencing. The protocol breaks down into five separate steps. (1) Preparation of trophozoite
suspension from frozen infected venous blood; (2) Characterization of nucleus-stained trophozoites by flow cytometry; (3) Isolation of trophozoites using an automatic cell sorter device; (4) Trophozoite whole genome amplification; (5) Applications: a- single-trophozoite microsatellite genotyping or, b- whole genome sequencing.

Preparation of trophozoite suspension from frozen blood samples
To optimize the following protocol, several protocols described in previous studies [13-16] were combined. Trophozoite isolation was carried out using a 40 μL sample of Plasmodium-infected venous blood added to 200 μL of NH4Cl-KHCO3 buffer (0.15 M NH4Cl, 1 g/L KHCO3, Ratio 1:5 v/v), which induces the bursting of red blood cells. The mixture was kept on ice for 5 min. Following centrifugation at 15,129g for 5 min at 4°C, the supernatant was discarded. The sample was then centrifuged again at 1,432xg for 15 min at 4°C, the supernatant discarded, and the pellet re-suspended in 200 μL of Buffer I (15 mM NaCl, 0.34 M Sucrose, 0.2 mM EDTA, 0.2 mM EGTA, 15mMTris-HCl pH7.4, 0.15 mM Spermine, 0.5 mM Spermidine, with one tablet from the Complete kit (Roche, Bazel, Switzerland) to avoid DNA damage). The sample was then centrifuged at 1,432xg for 15 min at 4°C, the supernatant discarded and the pellet re-suspended in 200 μL of Buffer I + 1% (v/v) TritonX-100 to remove disrupted cell membranes. The sample was centrifuged again at 1,432xg for 15 min at 4°C and the supernatant was discarded. 200 μL of Buffer I was added to eliminate Triton and maintain DNA integrity. The solution was finally centrifuged at 1,432xg for 15 min at 4°C, the supernatant discarded and the pellet re-suspended in 100 μL of Phosphate Buffered Saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.76 mM KH2PO4), with 1 μL of RNaseA (10 μg/mL) to eliminate RNA to avoid cross fluorescence. Finally, the preparation was stored on ice to maintain a monodispersed suspension of cells.

Characterization of nucleus-stained trophozoites by flow cytometry
DNA was stained with Propidium Iodide (PI) at a 10 μg/mL final concentration [14] in a 1.5 mL microtube and kept for at least 1 h in the dark. The stained samples were then analysed with a FACSAria (Becton-Dickinson, Mountain View, CA). The PI-stained nuclei were excited with a 488 nm light and were gated on the basis of their fluorescence intensity (FI) and forward scatter (FSC) [17]. Logarithmic red fluorescence was detected through a 585/42 nm band pass filter (FL2). The speed of sorting was 5,000 events/sec and for each sample, 50,000 cells were acquired, stored and analysed using the FACSDiva software (Becton-Dickinson, Mountain View, CA). The trophozoite area was defined in a two-dimensional scattergram of PE-H (PhycoErythrin-H), which corresponds to fluorescence intensity and FSC (Forward Scatter) primarily by comparing scattergrams for parasite-positive and parasite-negative blood samples (Figure 1). The relative position of the trophozoites and the human cells was determined by comparing cultured parasites with an uninfected blood sample.

Isolation of trophozoites using an automatic cell sorter device
Trophozoites were then sorted using an Automatic Cell Deposition Unit (ACDU) and placed in standard 96-well plates. Cell sorting was done for 1 and 200 trophozoites per well. Our sorting mode was optimized for maximal purity to ensure that only one target event would be sorted. Each well contained 9 μL of the sample buffer, which was subsequently used for whole-genome amplification (see below). During the entire process, trophozoite solutions were always kept on ice.

Trophozoite whole genome amplification
Whole genome amplification was carried out using the “Illustra GenomiPhi™ V2 DNA Amplification Kit” (GE Healthcare, Uppsala, Sweden) following the manufacturer’s instructions. The trophozoite preparation was denatured by heating at 95°C for 3 min, and then kept on ice. 9 μL of Reaction Buffer (Illustra™ GenomiPhi™ V2 DNA Amplification Kit” - GE Healthcare, Uppsala, Sweden) and 1 μL of enzyme Phi 29 was added, and the preparation incubated at 30°C for 2 h for genome amplification. The amplification was stopped by placing the samples at 65°C for 10 min, before being stored at −20°C.

Applications
Single cell microsatellite genotyping
Single trophozoite whole genome amplified DNA was used as a template for PCR-based amplification of seven polymorphic microsatellites loci (POLYa, TA60, ARA2, Pfg377, PiPK2, TA87, TA109) distributed on five of the 14 Plasmodium falciparum chromosomes as listed in Anderson et al. [4,5]. A two-step semi-nested PCR strategy with fluorescent end-labelled primers was used for the microsatellite amplification, as described in Razakandrainibe et al. [18], following the methodology developed by Anderson et al. [4]. The amplified microsatellite repeats were resolved and sized relative to an internal size standard using a Genetic Analyzer 3130xl and the GeneMapper software (Applied Biosystems, Carlsbad, CA). As a positive control, total DNA was genotyped. Each DNA sample was isolated and purified using the DNeasy blood and tissue kit® (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

To perform this study a total of seven blood samples collected from six infected patients in Senegal (see...
Table 1, Patients 2–7) and one from The Republic of the Congo (Table 1, Patient 1) were used. As a positive control, an in vitro culture of *P. falciparum* trophozoites (strain 3D7) was used. A non-infected blood sample was also used as a negative control. For each sample, 10 single trophozoites were sorted and genotyped.

Real time PCR was used to determine the parasitaemia of each sample following the protocol described by Bourgeois and colleagues [19,20].

**Plasmodium falciparum** DNA enrichment and whole genome sequencing

The infected venous blood of one patient from Senegal (patient 8, Table 1) was used. Parasitaemia was estimated by Real Time quantitative PCR to be around 4,200 parasites/μL. Human blood contains between 5,000 to 7,000 diploid white nucleated cells per μL, and the haploid human genome is approximately three billion base pairs and that of *P. falciparum* around 23 million base pairs. Accordingly we estimate human DNA to be between 310 and 434 times more prevalent in the total DNA extract than *Plasmodium* DNA.

To enrich the *Plasmodium* DNA preparation, 200 trophozoites were twice sorted from the blood sample (following the protocol described above) and whole genome amplification separately performed on these two sets of trophozoites. The resulting amplified DNA from these replicate genome amplifications were pooled for

**Figure 1** Isolation of *Plasmodium falciparum* trophozoites by flow cytometry. On the left panel, Figures 1a, 1b, and 1c represent a two-dimensional scattergram which corresponds to low-angle light scatter (FSC-A) as a function of fluorescence intensity (Phycocyanin, PE-H). Logarithmic red fluorescence was detected through a 585/42 nm band pass filter (FL2). The speed of sorting corresponded to 5,000 events/sec and for each sample, 50,000 cells were acquired, stored and analysed. The speed of sorting corresponded to 5,000 events/sec and for each sample, 50,000 cells were acquired, stored and analysed. Note that the scales of these figures are not the same. P1 window (blue) represents the trophozoite population (the negative control contains debris). P2 window (pink) indicates the position of white blood cells. Figure 1a shows an uninfected blood sample used as a negative control. The corresponding histogram (Figure 1d) shows the distribution of debris. In the middle panel (Figure 1b) the scattergram of the population of parasite stages found in a *P. falciparum* 3D7 culture is shown (Figure 1b). The corresponding histogram (Figure 1e) shows the distribution of trophozoites in the P1 zone. The lower panels (Figure 1c and Figure 1f) represent the parasitaemia of an infected field sample (patient 2 – Table 1).
subsequent sequencing to limit potential misrepresentation of certain parts of the genomes due to a random, and not locus-specific, failure of amplification after WGA.

Whole genome sequencing was performed by Integragen (Evry, France) using a Genome Analyser GA II Illumina machine. Two libraries of fragmented DNA were prepared (average size of fragments: 330 bp) and 36 bases sequenced on both extremities. Sequencing was performed in two lanes, the first with the standard enzyme from the Illumina kit and the second with Herculase II Fusion (Agilent, Santa Clara, CA), an enzyme that reduces the amplification bias due to the AT-rich composition of the *P. falciparum* genome (~ 80% AT). Sequence data were analysed using the Illumina Pipeline and were performed by Integragen.

**Results**

**Isolation and trophozoite cell-sorting**

To isolate single trophozoites after blood sample extraction, the trophozoite population was characterized using two representative two-dimensional scattergrams. The scattergrams in Figures 1a, 1b and 1c represent fluorescence intensity (PE-H, PhycoErythrin-H) as a function of the forward low-angle light scatter (FSC-A), and the scattergrams in Figures 1d, 1e and 1f represent the distribution of the number of sorted cells (counts) as a function of PE-A (Phycoerythrin-A), which corresponds to propidium iodide fluorescence intensity. Figures 1a, 1b and 1c show different cell populations in venous blood samples after trophozoite extraction: ring stage trophozoite populations (P1, blue), nucleated white blood cell populations (P2, pink) and debris stained with Propidium Iodide. Each dot is considered as one event defined as trophozoite, white blood cell or debris. Debris may have different origins: damaged or fragmented host nuclei, aggregates of membranes where free DNA is fixed. Thus the presence of DNA causes their fluorescence and can be misdiagnosed as trophozoite nuclei (i.e. if present in the P1 zone). In addition to that, debris may correspond to background noise caused by the machine. This is more likely as we worked at the logarithmic scale to increase detection sensitivity (due to the small amount of DNA within trophozoite nuclei). For each treated sample; trophozoites were always sorted from the P1 window and isolated for genetics studies.

**Single-cell genotyping**

Single trophozoites were amplified at seven microsatellite loci. The average rates of amplification for each sample are shown in Table 1. On average, amplification success was 38%. Strong variations in amplification success were however observed among single-trophozoite samples (8% to 66% microsatellite amplification success).

**DNA enrichment and whole genome sequencing**

Two lanes of sequences were generated for each sample. One lane using the classic Illumina enzyme, and another the Herculase II Fusion enzyme, which reduces the amplification bias of AT-rich genomes such as that of *P. falciparum*. 12,455,400 clusters (passing filters) were obtained for the first lane and 9,713,500 for the second one. The percentage alignment to the *Plasmodium* reference genome was higher for the second lane probably due to higher amplification of the *Plasmodium* genome using the Herculase enzyme.

Despite an estimated initial ratio of human to *Plasmodium* DNA ranging from 310 to 434 to 1, the method gave a final DNA sample composed on average by 62% *Plasmodium* DNA, 20% human DNA and 18% DNA that did not blast with any human or *Plasmodium* reference genomes (Table 2). Using this method, the

| Enzyme used | Number of passing clusters | % of passing clusters | % aligning on the *P. falciparum* reference genome (3D7) | % aligning on the human reference genome (Hg18) | % foreign DNA |
|-------------|-----------------------------|-----------------------|----------------------------------------------------------|-----------------------------------------------|---------------|
| Illumina    | 12,455,400                  | 92.00                 | 55.53                                                    | 20.41                                         | 24.06         |
| Herculase   | 9,713,500                   | 93.00                 | 69.45                                                    | 21.12                                         | 9.43          |
| Average     | 11,084,450                  | 92.5                  | 62.49                                                    | 20.77                                         | 16.74         |
enrichment level, calculated as the initial human/Plasmodium DNA ratio over the final human/Plasmodium DNA ratio was between 939 and 1283.

Regarding the quality of the P. falciparum genome sequence produced, reads obtained across all lanes covered 99% of the genome and were used to assemble 80% of the P. falciparum genome. The average coverage was 43X.

Discussion
The aim of this paper was to test two possible applications of flow cytometry and cell sorting for malaria genetic studies: (1) single trophozoite genotyping and (2) trophozoite DNA enrichment for subsequent whole genome sequencing. The idea of the paper was to use protocols already described and published and to combine them to test for these two applications. It is important to note that the method presented here can certainly be improved for better yields. Note moreover that although the current protocol was optimized for sorting P. falciparum ring trophozoites, the flow cytometric parameters for sorting other parasite stages (schizonts, gametocytes) [21] or other species could be determined.

Single cell genotyping
It was demonstrated, for the first time, the possibility to genotype single trophozoites isolated from frozen clinical blood samples using different microsatellite markers. The average proportion of alleles that were successfully amplified from single trophozoites was 38%, but this varied strongly between samples (from 8% to 66%). Several factors may explain these variations. First, some trophozoites might not have been loaded properly into the wells during sorting. Second, this could be due to improper sorting itself [9]. Indeed, as previously noted, the trophozoite area (P1) in which cells were picked during flow cytometry and sorting comprises both trophozoites and debris. Consequently, debris can also be sorted, which results in no amplification. Finally, this could be due to issues related to the conservation of blood samples and DNA. Indeed, all samples were conserved frozen for a long time (many for more than 10 years) and have undergone recurrent freeze-thawing cycles which may have led to DNA degradation [22].

As observed by Frumkin et al. [23], it is likely that the amplification success rate would be higher with single cells extracted from fresh samples. In the case of malaria research, methods of sorting have already been developed to specifically deal with these kinds of samples (cultivated or short terms cultivated parasites) [9,14,24,25]. These methods could, in principle, also be adapted to perform single cell genotyping. This is particularly true for the method developed by Miao et al. [9] that allows the rapid isolation of single malaria parasite-infected red blood cells by cell sorting.

Single cell genotyping opens new possibilities for the characterisation of the different haplotypes present within mixed-infections. Although the amplification success obtained here is too low to allow proper determination of each haplotype, the technologies used in genetics for genotyping or sequencing are improving so rapidly it is likely that better yields could be obtained soon. The limits of the method for archival frozen samples will certainly be fixed by the level of degradation of DNA due to recurrent freeze-thaw cycles.

Plasmodium DNA enrichment and whole genome sequencing
A major challenge to sequencing clinical malaria samples using next generation sequencing is the abundance of “contaminating” human DNA. Removal of leukocytes and other components from infected blood samples is, therefore, an important prerequisite.

Methods have recently been proposed to deplete white blood cells from the blood of infected patients [10,26] for subsequent whole genome sequencing of Plasmodium isolates. But these methods only deal with fresh samples. The only method that currently deals with frozen samples is the method developed by Melnikov et al. [11] that purifies parasite DNA using hybrid selection, but this method requires a good knowledge of the target genome.

The method described provides a good alternative as it does not rely on particular knowledge of the target genome and can, in principle, be adapted to any parasite species as long as it is possible to distinguish it from the host nucleated blood cells. Especially since, the enrichment level obtained in this trial was very similar to that obtained using other current existing methods [10,11]. Thus, from an initial blood sample estimated to present a ratio of 1 Plasmodium nucleotide to 310–434 human nucleotides, we finally obtained a DNA product composed of ~3 Plasmodium nucleotides to every human one. Moreover, the quality of the assembled genome was high covering around 80% of the reference P. falciparum genome (3D7 strain) with a depth of about 43X.

Although promising, a complete test of the method is required to assess its sensitivity and yields using samples with different parasite loads.

Conclusions
To conclude, this method allows the isolation, genotyping and sequencing of P. falciparum trophozoites from archival clinical blood samples. A lot of these precious samples, stored in freezers all over the world, are currently overlooked for population genetic studies because of technical limitations (multiclonal infections and host
DNA contamination). This method can easily be adapted to other pathogens or other malaria blood stages (e.g. gametocytes). In addition, the fact that our method applies to frozen samples makes it usable in most endemic settings. Once blood samples have been collected, it is indeed easy to freeze them until processed. The development of such methods is important as it opens new avenues for the understanding of the dynamics of multiple infections in nature as well as their role for parasite evolution.

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

Acknowledgments
The authors thank the IRD, CNRS, UM-1, UM2 for financial support. This work was financed by ANR MGANE 2007 3SE1 012 and support by FP7 collaborative project Health-2007-2.3.2-9 MALVECBLock. We thank JF Trape and J Akiana for providing clinical blood samples from Senegal and Republic of the Congo, respectively.

Author details
1 Laboratoire MIVEGEC, UMR IRD CNRS 224 CNRS 5290 UMI 911 Av. Agropolis, 34934, Montpellier, Cedex 5, France. 2 Montpellier Bio Imaging, U847 IRB, 27 avenue du Professeur Grasset, Montpellier, France. 3 Laboratoire DIMP, CNRS UMR5235, Université de Montpellier 2, Place Eugène Bataillon, 34095, Montpellier, France.

Authors’ contributions
AB, CA, PD, FR, FP designed the study. AB, CA, PD, CD, FR, FP analysed the data. All authors put forward different ideas and contributed to the early draft. AB, CA, FP wrote the paper. All authors agreed the final draft. *AB and CA contributed equally to this work. All authors read and approved the final manuscript.

Received: 21 December 2011 Accepted: 10 April 2012 Published: 14 May 2012

References
1. Read AF, Taylor LH: The ecology of genetically diverse infections. Science 2001, 292:1099-1102.
2. de Roode JC, Helinski ME, Anwar MA, Read AF: Dynamics of multiple infection and within-host competition in genetically diverse malaria infections. Am Nat 2005, 166:531-542.
3. Bell AS, de Roode JC, Sim D, Read AF: Within-host competition in genetically diverse malaria infections: parasite virulence and competitive success. Evolution 2006, 60:1338-1371.
4. Anderson TJ, Haubold B, Williams JT, Estrada-Franco JS, Richardson L, Mollinedo R, Bockarie M, Mokili J, Mharakurwa S, French N, Whitworth J, Velegio G, Breckman AH, Nosten F, Ferreira MU, Day KP: Microsatellite markers reveal a spectrum of population structures in the malaria parasite Plasmodium falciparum. Mol Biol Evol 2000, 17:1467-1482.
5. Anderson T, Su XZ, Bockarie M, Lagog M, Day KP: Twelve microsatellite markers for characterization of Plasmodium falciparum from finger-prick blood samples. Parasitology 1999, 119:113-125.
6. Certain I, Stiby CH: Plasmodium falciparum: a novel method for isolating haplotypes in mixed infections. Exp Parasitol 2007, 115:233-241.
7. Stephens M, Smith NJ, Donnelly P: A new statistical method for haplotype reconstruction from population data. Am J Hum Genet 2001, 68:978-989.
8. Stephens M, Donnelly P: A comparison of bayesian methods for haplotype reconstruction from population genotype data. Am J Hum Genet 2003, 72:1162-1169.
9. Miao J, Li X, Cui L: Cloning of Plasmodium falciparum by single-cell sorting. Exp Parasitol 2010, 126:198-202.
10. Aubum S, Campino S, Clark TG, Dijimde AA, Zongo I, Pinches R, Manske M, Mangano V, Alcock D, Anastasi E, Maslen G, Macinnis B, Rockett K, Modiano D, Newbold C, Dourado OJ, Ouedraogo JB, Kwiatkowski DP: An effective method to purify Plasmodium falciparum DNA directly from clinical blood samples for whole genome high-throughput sequencing. PLoS One 2011, 6:e22213.
11. Melnikov A, Galinsky K, Rogov P, Fennell T, Van Tyne D, Russ C, Daniels R, Barnes KG, Bochicchio J, Ndiaye D, Sene PD, Wirth DF, Nussbaum C, Volkman SK, Birren BW, Grinke A, Neafsey DE: Hybrid selection for sequencing pathogen genomes from clinical samples. Genome Biol 2011, 12:R73.
12. Barberio HA, Hodges E, Green RE, Briggs AW, Krause J, Meyer M, Good JM, Maricic T, Johnson PL, Xuan Z, Rooks M, Bhattacharjee A, Bizuela L, Albert FW, de la Rasilla M, Fortea J, Rosas A, Lachmann M, Hannon GJ, Pálbo S: Targeted investigation of the Neandertal genome by array-based sequence capture. Science 2010, 328:727-732.
13. Tenastappen LW, Loken NR: Five-dimensional flow cytometry as a new approach for blood and bone marrow differentials. Cytometry 1998, 35:48-55.
14. Pattanapanyasat K, Thaithong S, Kyle DE, Udomsangpetch R, Yongvanitchit K, Hider RC, Webster HK: Flow cytometric assessment of hydroxypropylidene iron chelators on in vitro growth of drug-resistant malaria. Cytometry 1997, 27:84-91.
15. Loureiro J, Rodriguez E, Dori J, Santos C: Comparison of four nuclear isolation buffers for plant DNA flow cytometry. Ann Bot 2006, 98:679-689.
16. Ling J, Zhuang G, Tazon-Vega B, Zhang C, Cao B, Rosenwaks Z, Xu K: Evaluation of genome coverage and fidelity of multiple displacement amplification from single cells by SNP array. Mol Hum Reprod 2009, 15:739-747.
17. Grimberg BT, Erickson JJ, Smokoski RM, Jacobberger JW, Zimmerman PA: Monitoring Plasmodium falciparum growth and development by UV flow cytometry using an optimized Hoechst-thiazole orange staining strategy. Cytometry A 2008, 73:546-554.
18. Razakandrainibe FG, Durand P, Koella JC, De Meeuw T, Roussel F, Ayala FJ, Renaud P: Clonal population structure of the malaria agent Plasmodium falciparum in high-infection regions. Proc Natl Acad Sci U S A 2005, 102:17388-17393.
19. Elsayed S, Plewes K, Church D, Chow B, Zhang K: Use of molecular beacon probes for real-time PCR detection of Plasmodium falciparum and other Plasmodium species in peripheral blood specimens. J Clin Microbiol 2006, 44:622-624.
20. Bourgeois N, Bocquet P, Basset D, Daudieu-Enault C, Charachon S, Lachaud L: Comparison of three real-time PCR methods with blood smears and rapid diagnostic test in Plasmodium sp. infection. Clin Microbiol Infect 2009, 16:105-1311.
21. Chevalley S, Coste A, Lopez A, Pipy B, Valentin A: Flow cytometry for the evaluation of anti-plasmodial activity of drugs on Plasmodium falciparum gametocytes. Malar J 2010, 9(Suppl 1).
22. Ross KS, Haines NE, Kelly JP: Repeated freezing and thawing of peripheral blood and DNA in suspension: effects on DNA yield and integrity. J Med Genet 1990, 27:569-570.
23. Frumkin D, Wasserstrom A, Itzkovitz S, Harmelin A, Rechavi G, Shapiro E: Amplification of multiple genomic loci from single cells isolated by laser micro-dissection of tissues. BMC Biotechnol 2008, 8:17.
24. Whaan JM, Ritterhaus C, Ip SH: Rapid identification and detection of parasitized human red cells by automated flow cytometry. Cytometry 1983, 4:117-122.
25. Hair JD: Two-color flow cytometric analysis of the growth cycle of Plasmodium falciparum in vitro: identification of cell cycle compartments. J Histochem Cytom 1986, 34:1651-1658.
26. Sriprawat K, Kaewpongsri S, Suwanarusk R, Leimanis ML, Lek-Uthai U, Phyo AP, Srounou G, Russell B, Renia L: Effective and cheap removal of leukocytes and platelets from Plasmodium vivax infected blood. Malar J 2009, 8:115.