DNA microarrays for malaria

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DNA microarrays are a powerful tool for the analysis of RNA and DNA composition on a whole-genome scale. The first applications of this technology in parasitology are in place. This review examines the various approaches to *Plasmodium* transcript-profiling that are being adopted using DNA microarray analysis and discusses additional strategies for obtaining and collating information relevant to the search for drug and vaccine candidates in malaria.

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Mortality resulting from malaria has motivated research on the causative parasite *Plasmodium falciparum* as a high priority, eliciting a formal project to sequence the entire parasite genome. Access to all open reading frames (ORFs) by the research community is imminent, and a fully annotated genome will soon be available. Sequence function in itself has already become too complex to be dissected by the classical reductionist approach to molecular biology, where genes are analyzed individually. Now, functional genomics techniques (including bioinformatics, gene mapping, genome modifications, and large-scale transcript analysis) can be used to unravel the biological roles of malaria genes [1–8]. Specifically, DNA microarrays can simultaneously analyze expression of thousands of malaria genes in a single experiment [6–8]. Such techniques are powerful in general, particularly for malaria research, because of the limitations of molecular genetics in malaria systems [6].

Microarray-based expression analysis

DNA microarrays (constructed from pre-synthesized DNA) and DNA chips (involving *in situ* DNA synthesis using light-directed chemistry) contain high density microscopic arrangements of immobilized nucleic acids. Microarraying of double-stranded or single-stranded DNA fragments (dsDNA or ssDNA) on a glass microscope slide is being used extensively owing to the accessibility, cost effectiveness and proven success of this approach [9,10]. DNA chips are outside the scope of this review.

Microarrays have been constructed largely from pre-existing sequence information and are used for transcript profiling – the most common application of this technology. In some early studies, differential gene expression under various physiological conditions was monitored in *Arabidopsis thaliana* [11] and in human tumorigenic cell lines [12] with microarrays of full length cDNAs and expressed sequence tags (EST). *Saccharomyces cerevisiae* ORFs [13,14] and individual genes [15] were arrayed to dissect the yeast cell cycle on an unprecedented scale culminating in the arraying of the first entire genome on one glass slide. In other seminal studies, cDNA microarrays were also used to describe the transcriptional responses of human fibroblasts to serum [16], the alterations in human host-cell transcription induced by HIV-1 [17] and to hunt for disease-related expression patterns in human cancers [18–20]. In addition to transcript profiling, microarrays can also reveal differences in gene copy number (DNA composition) between two cells [21].

Microarray fabrication

The construction of dsDNA microarrays has been comprehensively documented (Box 1 and Box 2). Protocols are available from publications and web sites (Box 1d,e) to guide the parasitologist through the procedure, whether the starting template material for array construction is a plasmid library or a genomic template.

Three broad variations of DNA microarray are now being constructed in malaria laboratories worldwide:

- Random (shotgun) *P. falciparum* microarrays
  These have been constructed out of a mung-bean-nuclease-digested genomic (gDNA) library, using universal primers to flank the vector sequence and polymerase chain reaction (PCR) amplification of gDNA inserts [6]. Mung-bean nuclease preferentially cuts malarial DNA that flanks the coding region [22]. Such digestion facilitates the cloning of unique coding regions and avoids over-representation of flanking sequences or introns in the library. This reduces the 30 Mb genome of *P. falciparum* to ~6000 genes either as whole ORFs or exons [2]. Genes previously implicated in specific processes (such as host cell invasion and parasite cytoadherence, and the developmental transitions of sporozoite differentiation and gametocytogenesis) can also be arrayed by themselves or in addition to the genome-wide ORFs to serve as controls and points of reference. Clearly, the sequences of the arrayed gDNA fragments on a shotgun microarray are unknown, necessitating post-hybridization sequencing of interesting clones. Sequencing of just a few hundred genes that were upregulated or downregulated in gametocytes increased the collection of stage-specific transcripts in malaria by an order of magnitude [6].
- Gene-specific microarrays
  These are currently being developed from large sets of well-characterized gene sequence tag (GST) and

http://parasites.trends.com 1471-4922/02/$ – see front matter PII: S1471-4922(01)02153-5

Box 1. Websites of interest and microarray resources for parasitologists

(a) Yeast expression data
http://genome-www4.Stanford.EDU/cgibin/SGD/expression/expressionConnection.pl

(b) Malaria *Plasmodium* database
http://plasmodb.org/PlasmoDB.shtml

(c) Shot-gun malaria gDNA microarrays
http://derisilab.ucsf.edu/malaria

(d) DeRisis lab: Programs and protocols
http://www.microarrays.org

(e) Brown lab microarray guide
http://cmgm.stanford.edu/pbrown

(f) Stanford microarray data and links
http://genome-www4.stanford.edu/MicroArray/SMD/

(g) National Human Genome Research Institute
http://www.nhgri.nih.gov/DIR/LCG/15K/HTML

(h) Core facilities for microarrays
http://linkage.rockefeller.edu/wli/microarray/core.html

(i) Microarray market place
http://www.lab-on-a-chip.com/files/corepage.htm

(j) Microarray Gene Expression Database Group
http://www.mged.org/

(k) Pathogen genomics-worldwide
http://www.ebi.ac.uk/parasites/paratable.html

(l) Pathogen genomics-NIH
http://www.niaid.nih.gov/dmid/genomes/

(m) Malaria links
http://www.geocities.com/aaadeel/malaria.html

(n) Malaria reagent repository (MR4)
http://www.malaria.mr4.org/mr4pages/Index.html

EST libraries of *P. falciparum* [7,8]. Such arrays have the advantage of starting with known sequences. The representation of genomic sequences could be biased in EST sequences because the library is usually derived from a specific stage of the parasite life cycle. Nonetheless, an enormous amount of fresh information can be derived from such arrays [7,8]. It is now revealed that several genes are selectively transcribed during different parts of the erythrocytic cycle and during sporozoite formation [7,8].

- Double-stranded ordered arrays
Following the publication of full-length sequences of chromosomes 2 and 3, a physical map of annotated genes in a specific linear order is now available [23,24]. More chromosome sequences are soon to follow. Amplified genes from genome template using sequence-specific primers are being arrayed alongside other gDNA fragments to provide additional insights into transcriptional regulation mechanisms. Subsequent hybridizations could determine if neighboring genes can form functionally related groups and coexpress, thus providing evidence for 'operon-like' gene arrangements in *P. falciparum* [25]. The characteristics of gene search engines are expected to play a role in the representation of genes on the array.

Other approaches to malaria array construction will soon follow. In addition to traditional PCR-derived DNA, a fourth, powerful approach relies on long oligonucleotides for direct printing of microarrays [26,27]. This approach is simpler because it bypasses the laborious PCR, DNA purification and gel analysis steps of traditional array construction, and the exact sequence of the 70mer DNA strand is known for each spot. However, the long oligonucleotides have to be selected from the coding strand. If there is significant transcription of antisense RNA in malaria [4], it could be of interest to design and include long oligonucleotides from both strands of the genome.

In future, the most comprehensive and universally attractive arrays will contain not only recognizable coding regions but also all intergenic regions [28]. Sometimes, it is tempting to build small function-specific microarrays representing, for example, genes encoding a family of surface antigens, genes encoding structurally homologous proteins, genes encoding proteins from a subcellular organelle or genes expressed synchronously during parasite development. However, in the long term, such small arrays are expected to be of limited use in comparison with a whole-genome array. The functional relatedness and interconnectedness of genes can be surprising, unexpected and far-reaching. If certain genes, such as those encoding signaling molecules, transporters and chaperones, are absent from a microarray, then novel interactions and clusters will be omitted from the data, and groups of functionally related genes for any process or physiological condition would remain incomplete. The functions of at least half of the genes in the genome are unknown and leaving them out of a small array would be a disservice to functional genomics. Transcriptional relationships discovered inadvertently often raise important new questions, spark further investigation, and ultimately help to determine function [9].

Microarray hybridization and analysis

The gathering of microarray-based data begins with pair-wise comparisons of RNA (or DNA) from two cell populations. Information on sequence abundance from two cell types is converted into two different fluorescent-colored cDNAs by reverse transcription. After a single hybridization of the two fluorescently labeled cDNA populations to a microarray, the differential fluorescence corresponding to each gene present on the array can be analyzed. A well-trained experimentalist, working with a defined biological system, can readily reproduce a differential hybridization signal by twofold or more. Detection of

Box 2. DNA microarray construction

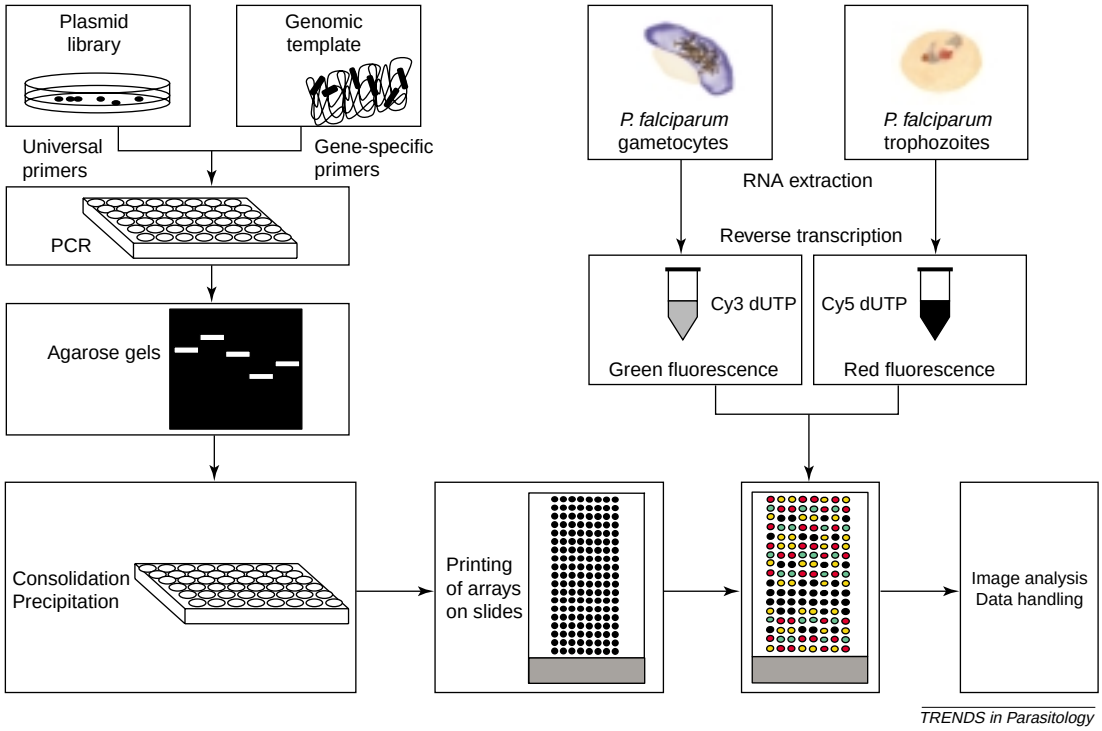
Double-stranded DNA (dsDNA) fragments are generated by PCR amplification and designated as probes (Fig. Ia). PCR products are evaluated by agarose gel electrophoresis, and probes of suitable size are consolidated into a 96-well format and robotically arrayed (printed) onto glass microscope slides. For each probe, the positions in a 96-well PCR plate and on the microarray are recorded. The DNA is then cross-linked to the slide. Once the microarrays are made, the researcher must identify a pair of distinct physiological states of the organism for analysis. RNA is collected from whole or fractionated cells, and the first strand cDNA is synthesized using oligonucleotide d(T) or random hexamers. Fluorescently labeled nucleotides are incorporated into the cDNA synthesis. Each pool of cDNAs is a complex mixture of target transcripts of unknown sequence (Fig. Ib). Target mixtures are added to the hybridization reaction with a microarray in equal quantities and the unbound cDNAs are washed away under appropriate stringency conditions. Scanning of the resulting fluorescent images with suitable excitation and emission filters using confocal microscopy constitutes the raw data from which differential gene expression ratios are calculated. The combination of these scanned images as a dual color image, which is normalized to compensate for differential efficiencies in labeling of cDNAs and detection of the fluors. The ratio of fluorescence at each gene on the array reflects differences in expression of that gene between the two cell types under investigation [a,b].

References

a Schena, M. *et al.* (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270, 467–470

b DeRisi, J. *et al.* (1996) Use of a cDNA microarray to analyse gene expression patterns in human cancer. *Nat. Genet.* 14, 457–460

Fig. I



transcripts is very sensitive: typically, 15 ml of *P. falciparum* culture at 3% parasitemia will provide 10 µg total RNA preparation. Starting with 10 µg of total RNA from each sample, it is possible to detect transcripts that are present at a concentration as low as one molecule per cell, and possibly lower [26,29].

An immense amount of numerical data from microarray experiments must be systematically collected, organized and analyzed. A series of software is freely available for these purposes on the web and is continually refined (Box 1d). Even before hybridization, programs such as GAL FILEMAKER allow specification of parameters such as spot size, spacing and orientation as thousands of DNA samples are transferred from 384-well plates to a glass slide during the arraying process. SCANALYZE processes fluorescent images of microarrays including semi-automated definition of grids and complex analysis of pixels and spots. Information from multiple experiments can be managed as flat file

databases designed specifically for microarray data (AMAD and NOMAD). These programs will run on any web server that supports Practical Extraction and Reporting Language (PERL). A mathematical description of similarity can be useful for finding genes whose expression patterns are shared [30]. CLUSTER will perform a hierarchical clustering, a pairwise average-linkage cluster analysis, to determine groups of genes according to expression level. The output (the biological relationships) can be displayed graphically as a pseudo-color matrix or a dendrogram using programs such as TREEVIEW.

Genes can cluster into functionally related groups, revealing the control mechanisms that dictate expression patterns of the genome. The greater the number of microarray hybridizations from which the data are taken (i.e. the more numerous the conditions under which an organism of interest has been sampled), the more strength is lent to cluster analysis [9,10]. Generally, redundant genes (those appearing more than once and varying in DNA size on the array) cluster together reproducibly, confirming that the exact positioning of a gene on an array makes little difference to its clustering properties and provides additional confidence in the expression data. Cluster analysis is therefore suitable for both random and gene-specific microarrays.

Microarrays without a full genome sequence?

The earliest malaria microarrays were constructed from *P. falciparum* mung-bean-nuclease-treated gDNA [6] (see Fig. I in Box 2). These early experiments established two key points related to the application of this technique to malaria research. First, despite seemingly low complexity of intergenic regions in malaria, *Plasmodium falciparum* displayed evidence for rich transcriptional regulation, the description of which was fully approachable using microarrays. Second, shotgun gDNA microarrays offered the possibility of an immediate, genome-scale experiment even in the absence of a complete *P. falciparum* genome sequence at that time. In order to calculate the number of individual clones necessary to make a comprehensive array, the redundancy incorporated into a plasmid library (original ligation mix) had to be calculated. *Plasmodium falciparum* pilot gDNA microarrays were constructed from the inserts of 3624 individual mung bean nuclease clones and assessed for redundancy by sequencing. From a subset of sequenced clones, ~60% were unique (appearing only once on the microarray). Extrapolating to a genome-wide scale, these data predict that a microarray constructed from the same gDNA library would require the printing of 10 000 cloned inserts to achieve >95% coverage of the *P. falciparum* coding sequence.

Microarrays for all *Plasmodium* species

For certain applications, shotgun gDNA microarrays can obviate the need for expensive, upfront sequencing of an entire genome. This is relevant for *Plasmodium* spp. whose genomes could remain largely unsequenced in the near future.

Given the limitations of experimentation in human hosts, *P. falciparum* is not always the optimum system for studying drug responses, immunological responses or host–parasite interactions. Other *Plasmodium* spp. are more easily manipulated in their vertebrate host and thus more experiments can be performed on the factors governing transmission. *Plasmodium berghei* and *Plasmodium chabaudi* (rodent malaria parasites), *Plasmodium knowlesi* (primate parasite) and *Plasmodium gallinaceum* (chicken parasite) have been used extensively as model systems to study various aspects of parasite biology and are used to further the most recent advances in malaria transformation technologies [31]. Because sequence information is limited, the genomes of these *Plasmodium* spp. would provide ideal candidates for retrieving exceptional quantities of transcriptional information using the shotgun microarray approach to functional genomics.

In addition, *P. falciparum* is not the only malaria parasite to have significant consequences for global communities. Human infections with *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* also result in high morbidity worldwide. It would be useful for the malaria research community to develop genome-wide expression analysis tools for these organisms in addition to *P. falciparum*. Mung bean nuclease libraries have been prepared for *P. berghei* [32] and *P. vivax* [33]. Currently, projects are under way for shotgun approaches to arraying the *P. berghei* genome (A. Waters, pers. commun.) and the *P. yoelii* genome (A. Vaidya, pers. commun.). As a mung bean library has also been constructed for *Drosophila melanogaster* [34], the interesting possibility arises that such libraries could be made from the genomes of many organisms, including the malaria vector *Anopheles* (K. Vernick, pers. commun.). Genomic DNA microarrays might therefore be constructed for any organism that does not have ESTs or genome sequences available.

Genome-wide questions for malaria

Malaria DNA microarrays will have several numbers of applications which will expand and diversify over time (see Fig. II in Box 3).

Cell cycle and life cycle regulation

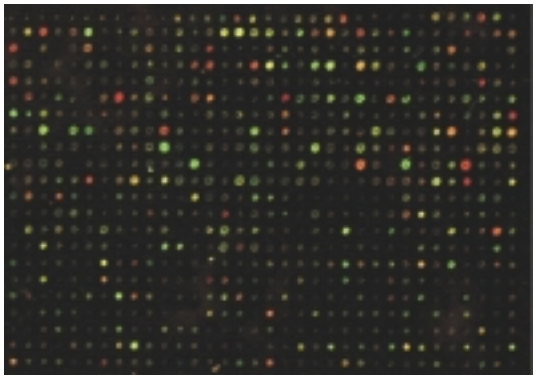
Several developmental milestones punctuate the *Plasmodium* life cycle. Each parasite stage is characterized by a transcriptional portrait (e.g. changes in glycolytic enzyme levels during intraerythrocytic development) [5,35]. The molecular mechanisms of control for transitions between

Box 3. Microarray applications

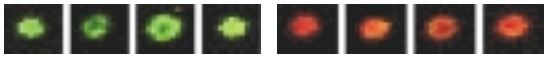
The hybridization data from a *Plasmodium falciparum* shotgun genomic DNA microarray is represented in Fig. I. Hybridization with asexual parasite cDNA (labeled with Cy3 dUTP; green fluorescence) and sexual-stage (gametocyte) cDNA (Cy5 dUTP; red fluorescence) are shown as a dual colour image (partial array shown in Fig Ia). A selection of genes from the array with the mung-bean clone identification number for each sequence and the induction ratio of expression (the ratio of fluorescence intensity of the stage-specific cDNAs to give a measure of transcript upregulation) (Fig. Ib). Abbreviations: GDH, glutamate dehydrogenase; HRP-1, histidine-rich protein 1; MSP1, merozoite surface protein 1; PEPCK, phosphoenolpyruvate carboxykinase; PfMRP, *P. falciparum* mitogen-activated protein kinase related protein; R45, antigen R45.

Examples of different malarial physiological states that could be probed with whole genome *Plasmodium* DNA microarrays and the types of problems that are expected to be solved from the output data are indicated in Fig. II.

(a)



(b)



Asexual stage transcripts Sexual stage transcripts

Clone I.D.	856	544	1382	2047	982	892	3268	1830
Name	HRP1	R45	GDH	MSP1	Pfg377	Pfs230	PfMRP	PEPCK
Induction ratio	13.97	2.06	4.24	4.59	23.11	5.41	3.26	7.52

Fig. I TRENDS in Parasitology

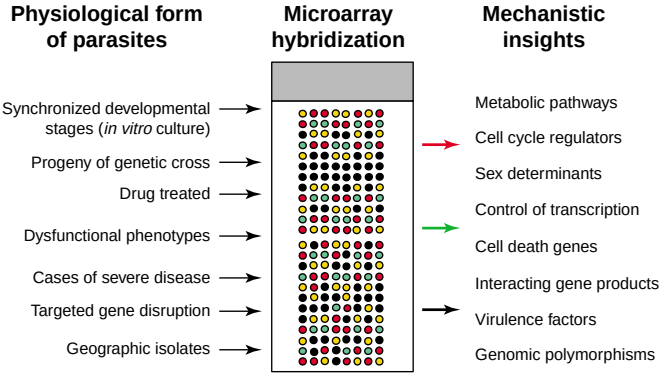


Fig. II TRENDS in Parasitology

discrete cell cycles are under investigation. Asexual intraerythrocytic parasites express stage-specific guanosine triphosphate hydrolases (GTPases), which are implicated in cell cycle control [36]. Commitment to sexual development might be designated by changes in trimeric G proteins [37] and groups of histone genes [38], and parasite development in the mosquito vector is delineated by two species of ribosomal subunit [39]. Stage-specific gene repertoires are being identified on a genome-wide scale by a developmental time-course analysis of transcripts using microarrays [6–8]. The transcriptional profiles from many points along the parasite life cycle will reveal all the genes that are functionally linked to those already characterized and will draw together the mechanisms by which *Plasmodium* transits through the vertebrate and insect hosts.

Transcriptional machinery

Molecular mechanisms of selective silencing of individual genes, in addition to their expression, are under scrutiny in *Plasmodium*. Traditional classes of transcription factors seem to be rare in the *P. falciparum* genome (G. Subramanian, pers. commun.) and there is no experimental evidence for their function. Several researchers propose that the chromatin structure influences the regulation of *var* gene transcription [40–42]. Interestingly, preliminary manipulations of histone deacetylases [43] could indicate the significance of chromatin remodeling in transcriptional control in general. Targeted gene disruption in *Plasmodium* spp. [31,44] will be fundamental for understanding gene regulation when coupled with microarray screening. Modification (either disruption or overexpression) of transcription factors and genes affecting chromatin structure might have genome-wide consequences, and the detection of which will be greatly facilitated with microarray experiments.

Mechanisms of drug action and drug resistance

Understanding the affects of current chemotherapies on *Plasmodium* is of paramount importance. The actions of even common drugs with defined targets, such as pyrimethamine and sulfadoxine, are poorly understood with respect to the cellular responses of the parasite and mechanisms of cell death. Rare events that lead to parasite survival

and eventual drug resistance are even more of a mystery. Other drugs, such as chloroquine and mefloquine, are likely to have even more complex modes of action. DNA microarrays provide an inexpensive platform for assaying the multiple effects of single and cumulative drug challenge, and accelerated drug resistance phenotypes [45] at the whole-genome level. Multiple experiments performed over, for example, a drug-exposure time-course are conveniently accomplished with microarray technology. Such time courses are important for avoiding artefacts because transcription is a dynamic process and a single 'snapshot' in time could give a limited impression of transcriptional responses.

Mapping transcriptional inheritance

An additional tool for studying complex parasite biology is inheritance. Classical genetics has given rise to several candidate genes for chloroquine resistance in *Plasmodium* [46,47]. The progeny of a genetic cross inherit chloroquine resistance primarily as a Mendelian trait [48]. Microarray screening of these progeny could uncover important factors in mechanisms of drug action and resistance. Differential gene expression data can also be combined with linkage maps recently generated from microsatellite and restricted fragment length polymorphism markers (RFLP) for the *P. falciparum* genome [48]. These markers segregate into linkage groups corresponding to each of the chromosomes. Quantitative trait analysis can be performed using these markers to identify loci that determine key parasite phenotypes such as drug resistance. Microarrays can complement genetic maps by determining the function of specific genes within that locus linked to a particular phenotype. In addition, by treating each transcriptional difference on a microarray as a phenotype, it should be possible to map the determinants of transcriptional differences between the parents of a genetic cross.

Identification of vaccine targets

All candidate antigens for *P. falciparum* vaccine development identified to date are expressed in a parasite stage-specific manner. Therefore, detailed description of stage-specific transcription will offer several new genes, which are candidate vaccine components. In addition to bioinformatics and large-scale protein analysis (proteomics), specific hybridization experiments can be designed to identify secreted or membrane-bound proteins. To do this, target cDNAs are generated from membrane-associated polysomes [49].

Virulence determinants

Cytoadherence of *P. falciparum* to host cells is a determinant of parasite virulence [50–52]. *P. falciparum* clones change expression of cytoadherance proteins *in vitro* and *in vivo* in response to available host cells or host proteins. Microarray analysis will help explore the role of individual transcripts of cytoadherance genes in such processes and also to identify global control mechanisms that govern changes in expression of virulence factors. The repertoires of transcripts from parasites taken from a range of patients and primate models [e.g. with mild or severe disease, drug treated or splenectomized (P. David, pers. commun.)] can be compared between clinical settings and also with parasites adapted to *in vitro* culture. This kind of analysis might also reflect the biology of the vertebrate host.

Future technical challenges

Generally, only small quantities of *Plasmodium* RNA can be obtained owing to constraints in the species and life cycle stage of the parasite that can be cultured. Intraerythrocytic stages of *P. falciparum* can be grown in the laboratory, but RNA from the rest of the life cycle must be salvaged from the mosquito vector or liver cells. Similarly, the other *Plasmodium* spp. can be, at best, partially maintained *in vitro*, restricting harvests from animal passages and field samples to small yields. This could potentially impede a microarray analysis because 8–10 μg of total RNA is required from each sample for a single experiment at present [6]. However, global single cell reverse transcription-polymerase chain reaction (GSC RT-PCR) appears to be a promising technique with applications to *Plasmodium*. cDNAs amplified by GSC RT-PCR represent most of the mRNAs from a single cell, including those of low abundance and in relative proportion to the original mRNA starting material [53]. Relative transcript levels between two cells can also be compared within a fivefold difference. Faithful amplification of all parasite transcripts using GSC RT-PCR would compensate for the low parasite numbers available.

Perspective

Microarray technology is an extremely powerful tool for the study of gene transcription and the relatedness of genes by function. Microarray data will complement the genome sequence information available for *Plasmodium* and for many other pathogens (Box 1k,l). The scale on which a parasite genome can now be analyzed is unprecedented. Integration of microarray data with proteomics will add to our understanding of molecular mechanisms of regulation and adaptation further.

Initially, the unfamiliarity of investigators with the details of microarray technology necessitates collaborations, but it will not be long before every parasitologist will have ready access to this technique in a reliable and affordable form following

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the rapid progress of microarray technology into most major research centers (Box 1h). In addition, in the near future, MR4 [the key reagent repository for malaria (Box 1n)] plans to distribute quality controlled microarray reagents to malaria investigators worldwide.

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