A shotgun optical map of the entire *Plasmodium falciparum* genome

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The unicellular parasite *Plasmodium falciparum* is the cause of human malaria, resulting in 1.7–2.5 million deaths each year[1]. To develop new means to treat or prevent malaria, the Malaria Genome Consortium was formed to sequence and annotate the entire 24.6-Mb genome[2]. The plan, already underway, is to sequence libraries created from chromosomal DNA separated by pulsed-field gel electrophoresis (PFGE). The AT-rich genome of *P. falciparum* presents problems in terms of reliable library construction and the relative paucity of dense physical markers or extensive genetic resources. To deal with these problems, we reasoned that a high-resolution, ordered restriction map covering the entire genome could serve as a scaffold for the alignment and verification of sequence contigs developed by members of the consortium. Thus optical mapping was advanced to use simply extracted, unfractionated genomic DNA as its principal substrate. Ordered restriction maps (*Bam*HI and *Nhe*I) derived from single molecules were assembled into 14 deep contigs corresponding to the molecular karyotype determined by PFGE (ref. 3).

Optical mapping is now a proven means for the construction of accurate, ordered restriction maps from ensembles of individual DNA molecules derived from a variety of clone types, including bacterial artificial chromosomes[4] (BACs), yeast artificial chromosomes[5] (YACs) and small insert clones[6]. We previously developed approaches for mapping clone DNA samples that relied on the analysis of large numbers of identical DNA molecules. Here, the challenge was to develop ways to generate restriction maps of a population of randomly sheared DNA molecules directly extracted from cells that were obviously non-identical. Problems to be solved included the development of techniques for mounting very large DNA molecules onto surfaces and new methods for accurately mapping individual molecules, which were uniquely represented within a population. Finally, new algorithms were necessary to assemble such maps into gap-free contigs covering all 14 chromosomes of the *P. falciparum* genome.

We developed an optical mapping approach, termed shotgun optical mapping, that used large (250–3,000 kb), randomly sheared genomic DNA molecules as the substrate for map construction (Fig. 1*a*–*e*). Random fragmentation of genomic DNA occurred naturally as a consequence of careful pipetting and other manipulations. Surface-mounted molecules were digested using *Bam*HI and *Nhe*I (refs 6–8). Because genomic DNA molecules frequently extended through multiple digital image fields, we developed an automated image acquisition system (GenCol) to overlap digital images with proper registration (Figs 1*c* and 2). Map construction techniques were altered to take into account local restriction endonuclease efficiencies (the rate of partial

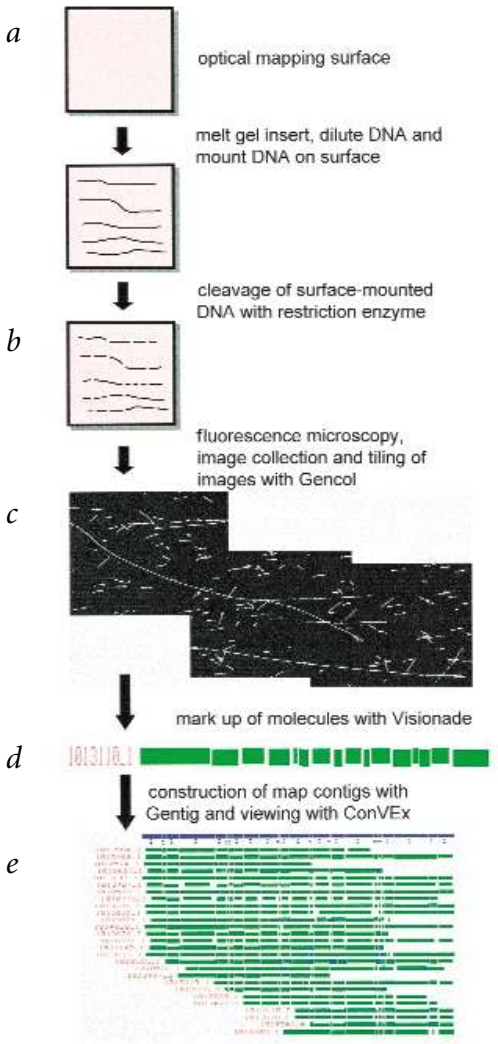

Fig. 1 Schematic of shotgun optical mapping approach. ***a***, Shotgun optical mapping used large (250–3,000 kb), randomly sheared genomic DNA molecules as the substrate for map construction. ***b***, Random fragmentation of genomic DNA occurred naturally as a consequence of careful pipetting and other manipulations. Surface-mounted molecules were digested using *Bam*HI and *Nhe*I (ref. 8). ***c***, Because genomic DNA molecules frequently extended through multiple image fields, an automated image acquisition system was developed (GenCol) and used to overlap images with proper registration. ***d***, Map construction techniques take into account local restriction endonuclease efficiencies (the rate of partial digestion) and the analysis of molecule populations that differed in composition and mass. ***e***, These steps were necessary to enable accurate construction of map contigs.

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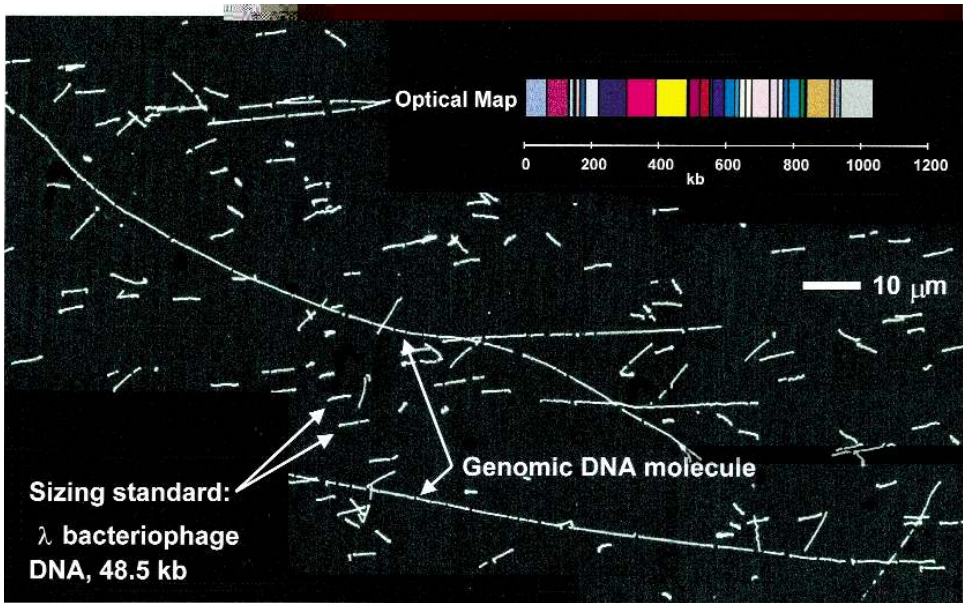

Fig. 2 Digital fluorescence micrograph and map of a typical genomic DNA molecule. A *P. falciparum* molecule digested with *Nhe*I is shown with its corresponding optical map. Comparison with the consensus optical map shows this molecule to be an intact chromosome 3. Image composed by tiling a series of 63× (objective power) images using GenCol. Co-mounted λ bacteriophage DNA is used as a sizing standard and to estimate cutting efficiencies.

digestion) and the analysis of molecule populations that differed in composition and mass. These steps were necessary to enable accurate construction of map contigs.

Previous map construction techniques using cloned DNA molecules[5,6,9] determined restriction-fragment mass on the basis of relative measures of integrated fluorescence intensities or apparent lengths. Thus, fragment masses were reported as a fraction of the total clone size (1.0), and later converted to kilobases by independent measure of clone masses (that is, cloning vector sequence[10]). Additionally, maps derived from ensembles of identical molecules were averaged to construct final maps. In contrast, here, we independently sized restriction fragments in genomic shotgun optical mapping using λ bacteriophage DNA that was co-mounted and digested in parallel (Fig. 2). These molecules were also used to locally monitor the restriction digestion efficiency, and to infer the extent of digestion on a per molecule (genomic) basis. Cutting efficiencies were in excess of 80%. This assessment provided a critical set of parameters for the contig assembly program, 'Gentig'[8,11,12], to reliably overlap maps derived from individual DNA molecules.

Gentig assembled maps into a number of deep contigs, but did not assign every single-molecule map to a contig. The program assembled contigs using 50% of the available molecules, which corresponded to 70% of the total mass of the molecules. In other words, the program was better able to construct contigs from the longer single-molecule maps. Finishing work using spreadsheets assembled the data into 14 contigs corresponding to the PFGE-generated molecular karyotype, with a total genome size of 24.16 Mb (Table 1). *Bam*HI and *Nhe*I maps had an average fragment size of 30.6 kb and 30.1 kb, respectively. We constructed consensus maps (Fig. 3) by simple averaging of aligned restriction-fragment masses (typically 6–26 fragments) derived from overlapping DNA molecules. Overall, chromosome sizes were largely consistent with PFGE results, with the total optical genome size being approximately 7% smaller, indicating that no previously uncharacterized nuclear component was found.

We previously constructed a high-resolution optical map of *P. falciparum* chromosome 2 (ref. 7). The starting material was a PFGE gel slice containing fractionated chromosome 2 DNA. We now constructed a whole-genome optical map using total, unfractionated genomic DNA as the starting material and resolved all 14 chromosomes, including electrophoretically unseparable ones (chromosomes 5–9, termed the 'blob'), at the level of data (optical map contigs) rather than as physical entities (that is, gel bands).

Table 1 • *P. falciparum* whole-genome optical mapping

Chr.	PFGE (Mb)	*Nhe*I (Mb)	*Bam*HI (Mb)	Ave. (Mb)	Diff. (Mb)	Linkage/ confirmation	Orientation
1	0.65/0.65*	0.684	0.668	0.676	0.016	1,3	+
2	1.0/0.947*	0.958	1.037	0.997	0.079	1,3	+
3	1.2/1.060*	1.084	1.096	1.090	0.012	1,2	+
4	1.4	1.311	1.306	1.309	0.005	1	
5	1.6	1.331	1.337	1.334	0.006		
6	1.6	1.395	1.373	1.384	0.022		
7	1.7	1.494	1.444	1.469	0.050		
8	1.7	1.495	1.504	1.499	0.009		
9	1.8	1.600	1.595	1.598	0.005		
10	2.1	1.808	1.688	1.748	0.120	1	
11	2.3	2.097	2.089	2.093	0.008	1	
12	2.4	2.478	2.361	2.419	0.117	1	
13	3.2	3.172	3.022	3.097	0.150	2	+
14	3.4	3.436	3.404	3.420	0.032	1,3	+
Total	26.05	24.341	23.974	24.157	0.367		

*Size from sequencing. Linkage/confirmation was obtained as follows: by mapping PFGE-purified chromosomal material (1); by mapping chromosome-specific YACs (2); or by sequence information (3). +, *Bam*HI and *Nhe*I maps have been oriented. Chr., chromosome; Ave., average size; Diff., difference between *Bam*HI and *Nhe*I maps.

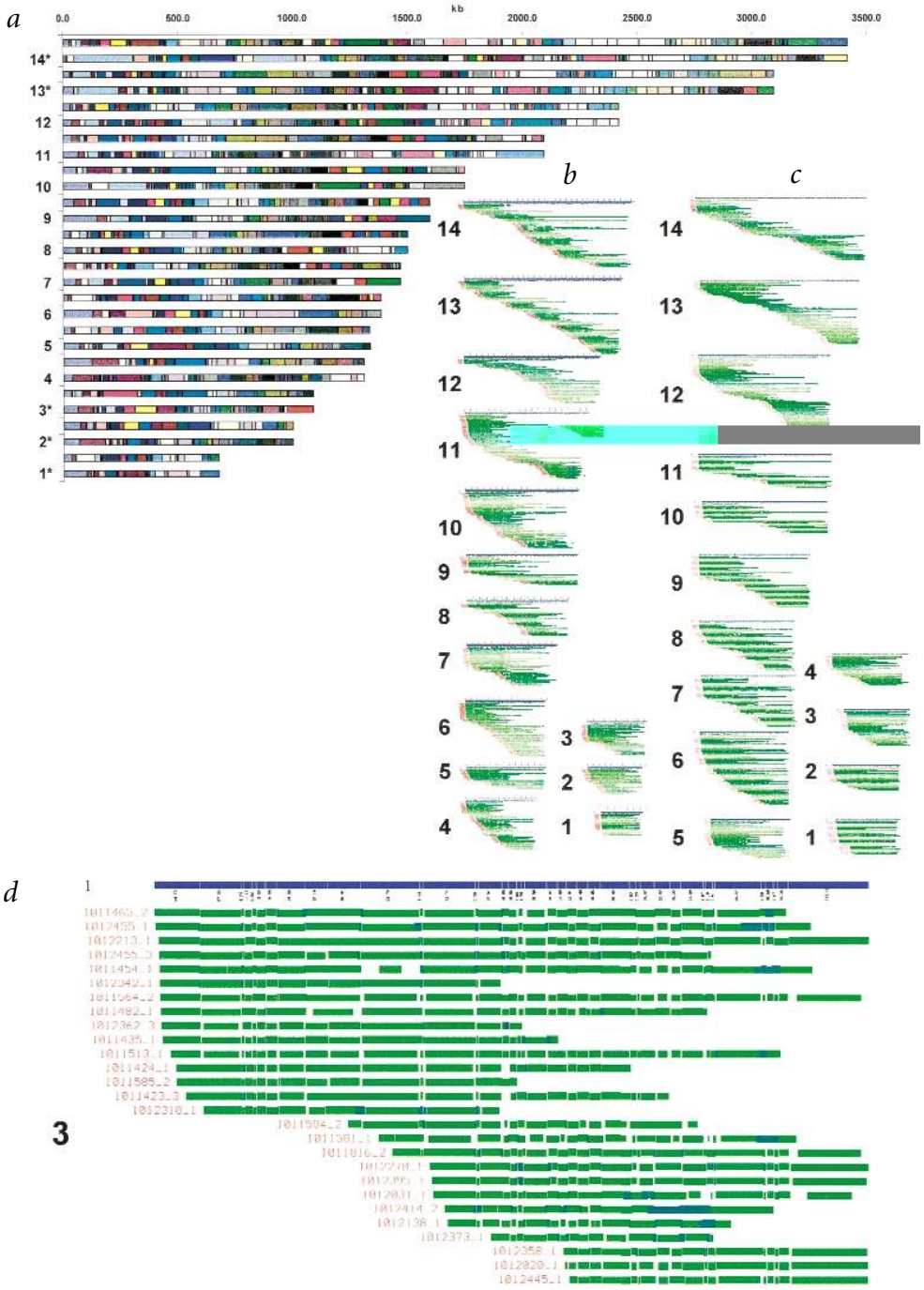

Fig. 3 High-resolution optical mapping of the *P. falciparum* genome using *Nhe*I and *Bam*HI. We mapped 944 molecules with *Nhe*I; the average molecule length was 588 kb, corresponding to 23× coverage. We mapped 1,116 molecules with *Bam*HI; the average molecule length was 666 kb, corresponding to 31× coverage. ***a***, Gap-free, consensus *Nhe*I and *Bam*HI maps were generated across all 14 *P. falciparum* chromosomes using the map contig assembly program Gentig. ***b***,***c***, *Nhe*I and *Bam*HI map alignments determined by Gentig, displayed by ConVEx. Fragment sizes of consensus maps (blue lines) shown in (*a*) were determined from the alignment and averaging of maps derived from 6–26 underlying individual molecules (green lines), 230–2,716 kb. ***d***, Enlargement of contig for chromosome 3 (*Nhe*I) shown in ConVEx displays maps (green) scaled to the consensus map (blue). These data can be accessed at http://carbon.biotech.wisc.edu/plasmodium. Bar lengths reflect measured fragment sizes. Fragments that overlap are shaded.

To assess errors produced by shotgun optical mapping, we compared optical restriction maps for chromosome 2 with restriction maps generated *in silico* using a previously assembled sequence[13]. We found good correspondence between the two maps. The sequence shows chromosome 2 to be 947 kb versus 958 kb by optical mapping with *Nhe*I and 1,037 kb with *Bam*HI. Only one 600-bp *Bam*HI fragment was missing in the entire genome optical map. The *Nhe*I optical map included all fragments above 400 bp predicted from sequence. The average absolute relative error in sizing fragments was 4.6% for *Nhe*I and 5.0% for *Bam*HI. Likewise, similar errors for chromosome 3 were determined by comparing optical maps with sequence data (*Nhe*I, 4.4%; *Bam*HI, 4.1%; total optical size versus sequence, *Nhe*I, 1,084 kb; *Bam*HI, 1,147 kb; versus 1,060 kb; D. Lawson, pers. comm.). These sizing errors were similar to those associated with PFGE.

Some large *Nhe*I and *Bam*HI fragments were noticeable at the telomeric ends. A telomere of one of the 'blob' chromosomes (chromosome 7) is composed of three consecutive 6-kb *Bam*HI fragments. Optical mapping can estimate numbers of repetitive regions if the repeats contain recognition sites for the endonuclease used. Subtelomeric regions in *P. falciparum*, however, are characterized by 21-bp tandem repeats[14], which are too small to be detected by optical mapping.

We used several approaches to verify and to link our optical maps with the PFGE molecular karyotypes, which number chromosomes according to mobility. Chromosomes that were identified and the orientations of *Bam*HI and *Nhe*I maps are shown

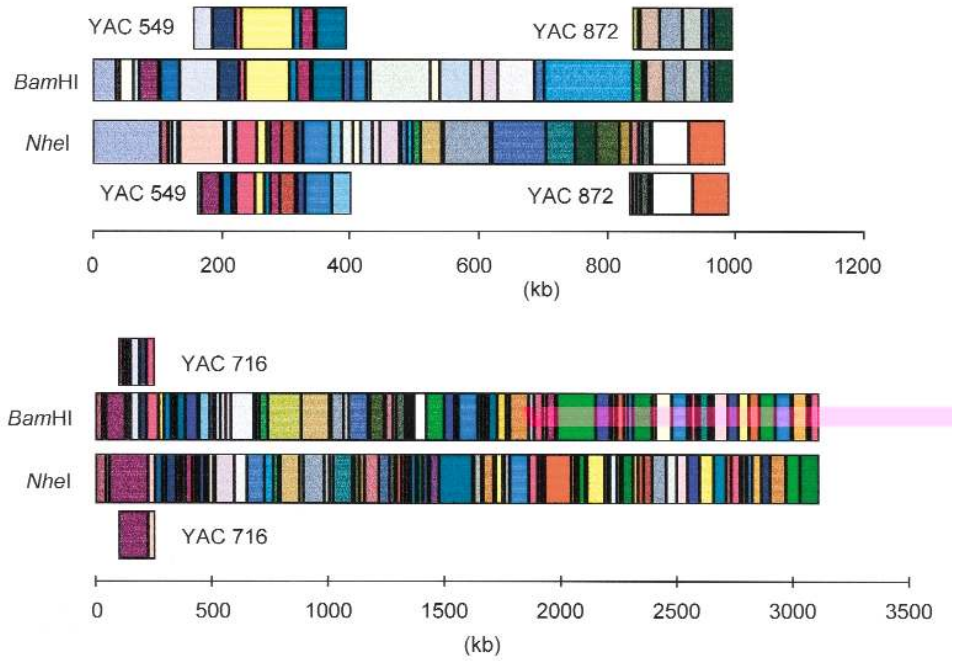

Fig. 4 Identification of chromosomes and alignment of *Nhe*I and *Bam*HI maps by mapping chromosome-specific YAC clones. Chromosome 3 and 13-specific YAC maps were aligned with the optical maps and the two enzyme maps were then oriented and linked. Each YAC is ~150 kb.

(Table 1). We confirmed chromosomal identities of some optical maps by optical mapping of PFGE-purified chromosomal DNA (ref. 7) with *Nhe*I or *Bam*HI. Here, most maps formed a contig, which aligned with a specific consensus map. Despite the fact that the largest and smallest *P. falciparum* chromosomes are resolved by PFGE, the gel slices contained DNA molecules from other chromosomes. There was, however, a sufficiently large population of molecules that formed a contig with a particular chromosome (>50%) to be able to identify it as being the chromosome predicted from the molecular karyotype. When many chromosomes are similar in size, such as chromosomes 5–9, there are many possible orientations of the maps, thus this approach was not viable. Chromosome-specific YAC clones were also optically mapped for further confirmation of chromosomal orientation and linkage. We aligned the resulting maps with a specific contig in the consensus maps (Fig. 4). YAC clones were not available for those chromosomes in the 'blob', so we were unable to identify or link these optical maps. As such, we have assigned numbers to these chromosomes according to their optically determined masses (Table 1). Maps can also be linked together by a series of double digestions, by the use of available sequence information or by Southern blot using chromosome-specific probes.

Because unicellular parasites have relatively small chromosomes that do not visibly condense, PFGE has provided a means by which chromosomal entities can be physically mapped and studied at the molecular level[15–17]. In fact, PFGE separations are currently providing the very material that the international malaria consortium is using to create chromosomal-specific libraries for large-scale sequencing efforts (http://www-ermm.cbcu.cam.ac.uk/dcn/txt001dcn.htm). Unfortunately, parasites such as *P. falciparum* can have karyotypically complex genomes, which confound PFGE analysis by displaying similarly sized chromosomes. Furthermore, very large or circular chromosomes are difficult to physically identify or characterize[18]. Although the shotgun sequencing of entire microorganism genomes[19,20] has obviated physical mapping to some extent, high-quality, finished sequence remains laborious to generate.

Many issues regarding the efficient sequencing of lower eukaryotes remain to be fully resolved, especially when available map resources are minimal. In the case of *Saccharomyces cerevisiae*, the entire genome was sequenced by a large consortium of laboratories on a per chromosome basis[21]. Their tasks were facilitated by the availability of extensive physical and genetic maps, plus an assortment of well-characterized libraries. These substantial genome resources provided ample means for the needed sequence verification efforts, and aids for the sequence-assembly process. In a similar, though much less distributive fashion, the *Caenorhabditis elegans* genome was recently completely sequenced[22]. Given the rapid pace of electrophoretic sequencing technology[23,24] and the accumulation of resources in sequence acquisition and analysis, new ways to efficiently sequence lower eukaryotes, particularly those implicated in human disease, must be developed to optimally leverage map resources created by optical mapping.

The optical maps presented here have been used by members of the consortium[13,25] as scaffolds to verify and facilitate sequence assemblies. In general, the maps were integrated into the sequence assembly process, in much the same way as any other physical maps. In particular, our maps have provided reliable landmarks for sequence assembly where traditional maps are somewhat sparse. Compared with sequence-tagged site (STS) or EST maps, in which landmark order is known but physical distance is approximate, optical restriction maps are constructed from landmarks (restriction sites) that are precisely characterized by physical distance. Another advantage is the speed of map construction: the maps presented here required only 4–6 months to generate. Given these and other advantages, future work will center on the algorithmic integration of high-resolution optical maps with primary sequence reads to more fully automate the sequence assembly and verification process. Finally, we plan to use optical mapping as the basis for developing of new ways to study genomic variations that fall between, or outside of, the capabilities of sequence-based approaches and cytogenetic observation.

Methods

Parasite preparation. We cultivated *P. falciparum* (clone 3D7) in erythrocytes using standard techniques[26]. Possible alterations of the genome that can occur in continuous culture[27] were minimized by keeping parasite aliquots frozen in liquid N_2 until needed. We then cultivated parasites only as long as necessary and prepared agarose-embedded parasites as described[7].

Mounting and digestion of DNA on optical mapping surfaces. We prepared derivatized glass optical mapping surfaces as described[7,28]. We diluted genomic DNA in TE buffer containing a sizing standard (λ bacteriophage DNA, 50 ng/ml), which was co-mounted with the genomic DNA by spreading the sample into the space between the surface and a microscope slide. DNA molecules were digested with *Nhe*I or *Bam*HI (ref. 8). λ bacteriophage DNA (48.5 kb; New England Biolabs) is cut once by *Nhe*I. λ DASH II bacteriophage DNA (41.9 kb; Stratagene) is cut twice by *Bam*HI. Therefore, we also used standards to identify regions on the surface where the digestion efficiency exceeded 70%. We stained DNA with YOYO-1 homodimer (Molecular Probes), before fluorescence microscopy. *P. falciparum* DNA has an AT content of 80–85%, and λ bacteriophage DNA has an AT content of 50%. The YOYO-1 fluorochrome used for DNA staining preferentially intercalates between GC pairs with increased emission quantum yield[29]. We therefore applied a correction factor to each fragment size to correct for this variation in fluorochrome incorporation.

Image acquisition, processing and map construction. We collected digital images of DNA molecules with a cooled charge coupled device (CCD) camera (Princeton Instruments) using Optical Map Maker (OMM) software as described[6]. Because genomic DNA molecules span multiple microscope image fields, we developed 'GenCol', an image acquisition and management software that was used to automatically collect and overlap consecutive CCD images with proper pixel registration. GenCol used a precise fitting routine, and the resulting 'super-images' covered the entire length of single DNA molecules, spanning several microscope fields. Restriction fragments were marked up with 'Visionade'[28], a semi-automatic visualization/editing program, which was run on super-images. Files created from marked-up images of molecules were then sent to map construction software, which automatically determined the restriction fragment masses, characterized internal DNA standard molecules and produced finished maps from single genomic molecules. The integrated fluorescence intensities of λ bacteriophage DNA standards, co-mounted with the genomic molecules, were used to measure the size of the *P. falciparum* restriction fragments on a per image basis. Cutting efficiencies (on a per image basis) were determined from scoring cut sites on sizing standard molecules contained in the same field as the genomic DNA molecules. Knowledge of endonuclease cutting efficiencies was critical for accurate contig construction.

Contig assembly by Gentig. Sophisticated statistical methods are used to overcome errors associated with partial digestion and mass determination[11,12]. Gentig finds overlapped molecules and assembles them into contigs. It computes contigs of genomic maps using a heuristic algorithm for finding the best scoring set of contigs (overlapping maps), because finding the optimal placement is in general computationally too expensive. The entire *P. falciparum* genome data set can be assembled into contigs in ~20 min. Gentig assembled consensus maps for each chromosome by averaging the fragment sizes from the individual maps underlying the contigs.

Contig viewing and editing by 'ConVEx'. We viewed contigs using 'ConVEx' (contig visualization and exploration tool). ConVEx is a multi-scale zoomable interface for visualization and exploration of large, high-resolution contiged restriction maps. Users can examine the consensus maps together with the raw uncorrected data. ConVEx also has a 'lens' mechanism that provides annotation and editing features, allowing communication of features such as STS markers, and even the underlying sequence reads.

Chromosome isolation by PFGE. The genome of *P. falciparum* is ~25 Mb, consisting of 14 chromosomes ranging from 0.6 to 3.5 Mb (ref. 28). PFGE resolves most of the *P. falciparum* chromosomes, except 5–9, which are of similar sizes and co-migrate. PFGE-purified chromosomal DNA was prepared as described[8] and used as a substrate for optical mapping.

YAC isolation and mapping. We cultured yeast cells in AHC media and prepared agarose-embedded cells using standard methods[3]. We purified YAC DNA using PFGE (POE apparatus, 1% gel in 0.5×TBE, pulse time 3 s, 5 s; switch time 32 s; 150 volts for 24 h; ref. 30). Optical maps of YAC clones were prepared with *Nhe*I and *Bam*HI as described above.

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