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Applications of optical DNA mapping in microbiology

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Optical mapping (OM) has been used in microbiology for the past 20 years, initially as a technique to facilitate DNA sequence-based studies; however, with decreases in DNA sequencing costs and increases in sequence output from automated sequencing platforms, OM has grown into an important auxiliary tool for genome assembly and comparison. Currently, there are a number of new and exciting applications for OM in the field of microbiology, including investigation of disease outbreaks, identification of specific genes of clinical and/or epidemiological relevance, and the possibility of single-cell analysis when combined with cell-sorting approaches. In addition, designing lab-on-a-chip systems based on OM is now feasible and will allow the integrated and automated microbiological analysis of biological fluids. Here, we review the basic technology of OM, detail the current state of the art of the field, and look ahead to possible future developments in OM technology for microbiological applications.

1. Optical mapping in microbiology: the beginning

Optical mapping (OM) is a technique capable of imaging single DNA molecules (Figure 1; Box 1). The use of OM in microbiology started in the 1990s as an auxiliary technique that, combined with Sanger nucleotide sequencing, supported reliable and cost-effective bacterial genome mapping (1). In 1999, Lin et al. (2) reported the first de novo shotgun OM-generated map of a microorganism, *Deinococcus radiodurans*. This map aided genome assembly (sequencing) as well as the discovery of new episomes and contributed to the elucidation of recombination mechanisms in this organism. Over the years, OM methods have been optimized, increasing the resolution and allowing smaller DNA fragments to be differentiated (generally in the kilobase range). While OM cannot fully replace most of the already established methods, it has been demonstrated that it is a good complementary or auxiliary method for two major applications: (i) comparative genome profiling, based on the detection of structural genome variations,

with applications in microbial typing; and, more recently, (ii) assembly and validation of whole-genome sequencing using high-throughput sequencing methods (Table 1). OM-based maps can be compared in silico with known sequences or, conversely, can be used as scaffolds for de novo assembly. These applications led to the recognition of OM restriction fragment mapping as a tool for rapidly identifying and/or characterizing microorganisms, motivating use of the technology for the development of commercial products (e.g., <http://opgen.com>; <http://bionanogenomics.com/>; www.genomicvision.com/).

1.1. First optical mapping-based technologies

In the first demonstration of OM, Schwartz et al. (3) used 4,6-diamino-2 phenylindole dihydrochloride (DAPI)-stained yeast DNA, molten agarose, and restriction enzymes. A flow across the surface of a microscope slide was used to stretch DNA to ~30% of its contour length, while gelation of the agarose fixed the DNA in the stretched conformation. Imaging the stretched DNA in a fluorescence

microscope at 37°C was combined with the addition of Mg²⁺ ions to activate the restriction enzymes. DAPI was shown not to inhibit restriction enzyme activation (4), and the resulting fragments were analyzed by direct measurement of the apparent length and the fluorescence intensity. This method worked well for fragments in the 0.2–1.0 Mb range, allowing creation of ordered restriction maps.

While the above method was promising, the thickness of the agarose led to problems with out-of-focus fluorescence. In addition, agarose is known to scatter light, thus reducing the signal. To improve image quality, Cai et al. (5) moved the system onto APTES (3-aminopropyltriethoxysilane)-treated glass coverslips, while Meng et al. (6) used polylysine-treated glass. By fixing DNA onto a surface after stretching with capillary flow, it was possible to hold the sample within the focal plane of a high numerical aperture objective, making focus easier to maintain. Immobilizing the DNA molecules eliminated the need for time-lapse imaging with agarose fixation. The authors found that despite DNA being bound to a glass surface using these methods, restriction enzymes were still able

Box 1

Optical mapping (OM) relies on the stretching of a DNA molecule (e.g. plasmid) or fragment. Stretching is achieved on positively charged or hydrophobic surfaces or by confinement in nanofluidic channels.

When stained with a fluorescent dye, it is possible to obtain an image of the stretched DNA molecule through fluorescence microscopy. To differentiate the nucleotide sequence patterns within the stretched DNA, labeling is required. Enzymatic labeling methods (e.g., restriction) are the most frequently used. Alternatively, methods based on the recognition of AT- and GC-rich regions through the utilization of specific dyes can also be used (e.g., denaturing mapping and competitive binding).

Images of the labeled stretched DNA molecules or fragments produce a characteristic pattern for a given molecule, and automated image-analysis software can determine the fragment sizes (Figure 1).

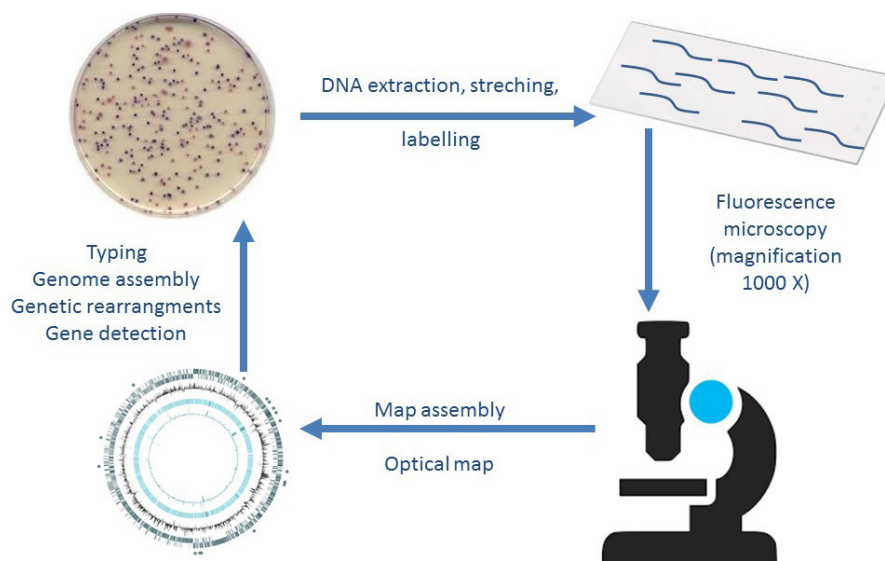


Figure 1. Overview of the main optical mapping (OM) steps and applications.

to function. In fact, because the DNA was stretched to 60% of its contour length on the glass compared with 30% in the agarose, access of the enzyme was improved, with an increase in the number of cut sites (5). Several improvements were subsequently made by Jing et al. (7), who showed the first automated OM system that used the “coffee-stain” effect to create spots containing discrete, individual molecules. Replacing ethidium homodimer or DAPI with YOYO-1 allowed use of higher salt concentrations and gave clearer images with higher contrast, thus improving the accuracy of fragment sizing (8). Machine learning was used to identify molecules and to create single-molecule maps that were then used for the construction of consensus maps.

In an early demonstration of its potential, OM was combined with the RecA-assisted restriction endonuclease (RARE) technique (9). Here, the enzyme RecA is combined with an oligonucleotide, driving the specificity of the enzyme to the complementary DNA sequence of the selected oligonucleotide. Since the RecA–oligonucleotide complex protects the binding site from methylation by cognate transferases, after removal of the RecA–oligonucleotide complex the non-methylated sites can be cleaved by a restriction endonuclease such as *EcoRI*. RARE allows the selection of specific regions for digestion and, hence, the mapping of specific genome regions.

1.2. DNA processing for OM analysis

Microfluidics enables reasonable throughput for OM, particularly the combination of microfluidics with advanced machine vision supported by adequate software systems

(10). These methods have been further improved with the development of the microchannel modality that allows oriented deposition of stretched DNAs, optimized analyte density, and synergy with machine vision technology. When it comes to molecular biology, important improvements were also made, since, as with any method that relies on the direct analysis of DNA, OM requires the recovery of high-quality DNA. The following sections summarize relevant aspects related to DNA extraction and digestion (Box 2).

DNA extraction. DNA extraction methods must be optimized for different cell types, such as yeast, Gram-negative/Gram-positive bacteria, or mycobacteria (11). The OpGen Sample Preparation Kit (OpGen, Inc., Gaithersburg, MD), which allows extraction of chromosomal DNA directly from isolated colonies or liquid cultures, has been widely used for high-molecular-weight genomic DNA preparation for OM (Table 1). The method was first reported by Schwartz and Cantor (12) and later modified for use in OM. It consists of a soft lysis to produce spheroplasts. The production of spheroplasts must be adequate for each type of cell envelope and can be facilitated by the use of enzymes or reagents such as lyticase, chitinase, zymolase, or glucucase for yeasts, or EDTA, N-lauroylsarcosine, or proteinase K for bacteria. Some bacterial cells, such as *Streptococcus*, *Lactobacillus*, or *Staphylococcus aureus*, may require additional lytic agents such as mutanolysin, endopeptidase-like lysostaphin, or others. Since procedures such as vortexing typically cause DNA shearing, they must be avoided, and

the separation of DNA from cell debris may require alternative methods such as magnetic beads or electrophoresis in low-melting-point agarose plugs (13,14). An interesting advantage of low-melting-point agarose plugs for DNA extraction is the possibility of storage prior to analysis without the risk of DNA degradation.

When the experimental goal is the OM of plasmid DNA, as described below in Section 2 (referring to emerging OM applications), the separation of plasmids from chromosomal DNA is required; thus, other DNA extraction methods are needed. Methods based on alkaline lysis, such as those used by commercial plasmid extraction kits (e.g., QIAGEN), have been successfully used for OM of plasmids with sizes up to at least 300 kb (15,16). These systems involve an anion exchange adsorbent onto which plasmid DNA selectively binds due to low-salt and pH conditions after alkaline lysis. RNA, proteins, metabolites, and other low-molecular-weight impurities are then removed by a medium-salt wash. Finally, supercoiled plasmid DNA is eluted in a high-salt buffer, with relatively high yields.

DNA digestion. Most OM microbiology applications described in the literature rely on the use of endonuclease restriction enzymes that cut DNA at specific short nucleotide sequences (Table 1). The number and size of fragments must be adjusted according to the purpose of the OM, the bacterial species, or the characteristics of the chromosomes. Rare-cutting enzymes produce large DNA fragments, typically greater than 100 kb, that are suitable for OM. However, even smaller PCR amplicons have been optically mapped

Table 1. Examples of optical mapping (OM) applications.

Objective	Species	Sample type	Other methods	DNA preparation	OM technique	Construction and analysis of OM	Reference
Bacterial identification	<i>Escherichia coli</i>	Culture collection	None	Conventional method	Competitive binding of YOYO* and netropsin**	In-house software	(58)
Comparative genomics	Shiga-toxicogenic <i>E. coli</i>	Clinical	PFGE; NGS	NA	Restriction (<i>NcoI</i>)***	OpGen software	(21)
	Enterotoxigenic <i>E. coli</i>	Clinical; Culture collection	SH	Genomic-Tip 100/G kit (QIAGEN)	Restriction (<i>Bam</i> HI, <i>Bsi</i> WI; <i>Eag</i> I)***	OpGen software	(27)
	Enterohemorrhagic <i>E. coli</i>	Clinical	NGS	Conventional method	Restriction (<i>NcoI</i>)***	OpGen software	(22)
	Shiga-toxicogenic <i>E. coli</i>	Clinical; Food-borne	NGS; MA; STGP	Gentle lysis and dilution	Restriction (<i>Bam</i> HI)***	OpGen software	(29)
	Enterohemorrhagic <i>E. coli</i>	Culture collection	None	Bacterial cells embedded into low-melting-point agar, further lysed and diluted	Restriction (<i>Bam</i> HI)***	OpGen software	(70)
	<i>Shigella flexneri</i>	Culture collection	None	Genomic DNA gel inserts further melted and treated with β -agarase	Restriction (<i>Bam</i> HI)***	In house software	(24)
	<i>Salmonella enterica</i> typhimurium	Clinical	PFGE; MLVA; PT	Bacterial cells embedded into low-melting-point agar, further lysed	Restriction (<i>NcoI</i>)***	OpGen software	(28)
	<i>Mycobacterium avium</i> spp. <i>paratuberculosis</i>	Culture collection	SS	Bacterial cells embedded into low-melting-point agar, further lysed; DNA gel inserts further melted and treated with β -agarase	Restriction (<i>Bs</i> WI) ***	Channel Collect; Pathfinder; OpGen Map Viewer	(26)
	Vancomycin-resistant <i>Enterococcus faecium</i>	Clinical	PFGE; NGS; MLST	NA	Restriction (<i>NcoI</i>)***	OpGen software	(20)
	Methicillin-resistant <i>Staphylococcus aureus</i>	Culture collection	SS	Bacterial cells embedded into low-melting-point agar, further lysed	Restriction (<i>Xba</i> I)***	Gentig software; OpGen Mapviewer	(25)
Typing	<i>Acinetobacter</i> complex	Clinical	None	OpGen Sample Preparation Kit; Agencourt Genfind v2 Kit	Restriction (<i>NcoI</i>)***	OpGen software	(68)
	Uropathogenic <i>E. coli</i>	Clinical; Culture collection	VFG; HA; PG; AR	NA	Restriction (<i>NcoI</i>)***	OpGen software	(23)
Genome assembly	Enterohemorrhagic <i>E. coli</i>	Culture collection	SS	Bacterial cells embedded into low-melting point agar, further lysed, and treated with proteinase K; DNA gel inserts further melted and treated with β -agarase	Restriction (<i>Nhe</i> I; <i>Xho</i> I) ***	Gentig software	(8) (71)
	<i>Yersinia pestis</i>	Culture collection	WGSGLS; PW	Genomic DNA gel inserts further melted and treated with β -agarase	Restriction (<i>Xho</i> I; <i>Pvu</i> II) ***	Gentig software	(32)
	<i>Yersinia aldovae</i>	Culture collection	NGS	NA	Restriction (<i>Afl</i> I) ***	OpGen software Open source software package#	(19)
	<i>Y. kristensenii</i>	Culture collection	NGS	NA	Restriction (<i>Bam</i> HI)***	OpGen software	(72)
	<i>Xanthomonas axonopodis</i> pv. <i>citrumelo</i>	Culture collection	NGS; SS	NA	Restriction (<i>Bam</i> HI)***	OpGen software	(72)
	<i>Xenorhabdus nematophila</i>	Culture collection	WGSGLS; PW	Bacterial cells embedded into low-melting-point agar, further lysed; DNA gel inserts further melted and treated with β -agarase	Restriction (<i>Eag</i> I; <i>Afl</i> I) ***	OpGen software	(38)
	<i>Xenorhabdus bovienii</i>	Culture collection	WGSGLS; PW	Bacterial cells embedded into low-melting-point agar, further lysed; DNA gel inserts further melted and treated with β -agarase	Restriction (<i>Eag</i> I; <i>Afl</i> I) ***	OpGen software	(38)
	<i>Rhodobacter sphaeroides</i>	Culture collection	NGS; SS	Genomic DNA gel inserts further melted and treated with β -agarase	Restriction (<i>Eco</i> RI; <i>Hind</i> III) ***	Gentig software	(73)
	<i>Rhodospirillum rubrum</i>	Culture collection	WGSGLS; PW	Genomic DNA gel inserts further melted and treated with β -agarase	Restriction (<i>Xba</i> I; <i>Nhe</i> I; <i>Hind</i> III) ***	ChannelCollect; Pathfinder; Gentig; OpGen Mapviewer	(37)
Genome structure	<i>Deinococcus radiodurans</i>	Culture collection	None	NA	Restriction (<i>Not</i> I; <i>Nhe</i> I) ***	Gentig software	(2)
	<i>Plasmodium falciparum</i>	Clone 3D7	PFGE	Agarose-embedded parasites and further lysed and treated with proteinase K; gel inserts further melted and treated with β -agarase	Restriction (<i>Nhe</i> I; <i>Bam</i> HI)***	GenCol; Visionade; ConVEx; Gentig software	(39)
Plasmid number and size	NA	Clinical	None	NA	YOYO*	In-house software	(59)

NA, not available; PFGE, pulsed field gel electrophoresis; NGS, next-generation sequencing; SH, subtractive hybridization; MA, microarray; STGP, Shiga toxin gene profile; MLVA, multilocus variable number of tandem repeat analysis; PT, phage typing; SS, Sanger sequencing; MLST, multilocus sequence typing; VFG, virulence factor genes; HA, hemolysis activity; PG, phylogenetic grouping; AR, antibiotic resistance patterns; WGSGLS, whole-genome shotgun library sequencing; PW, primer walking.

*, fluorescent DNA staining agent; **, antibiotic; ***, restriction enzyme.

#, <ftp://ftp.cbcb.umd.edu/pub/software/soma>.

(17). For OM applications, the restriction enzymes commonly used in OM-based microbial genome studies include *NotI*, *NheI*, *NcoI*, or *BamHI* (Table 1). However, it is not possible to identify a single enzyme that could be considered the best for analyzing all bacterial species genomes. Hence, it is advisable that for each species or genus, an adequate enzyme be selected. When a number of complete genome sequences is available, it is possible to optimize the selection of the target restriction site for a given species and therefore improve the potential of OM for typing. The optimal fragment sizes should be defined in accordance with the resolving power of the instrument and the requirements of the technique. A web-based tool (<http://insilico.ehu.es/>) developed by Bikandi et al. (18) allows the selection of restriction enzymes according to the DNA fragment sizes desired and the bacterial species under study. Restriction endonucleases that yield less than 50 fragments are useful for OM. *AbsI*, *AspA2I*, *XbaI*, and *PmeI* produce fragments of the desired size for *Klebsiella pneumoniae*, but not for *Pseudomonas* spp. strains, for which the endonuclease *SpeI* is adequate. This highlights the importance of the selection of an enzyme that targets suitable restriction

sites. Although newer OM applications may not rely on the use of endonuclease restriction (Section 2), the selection of suitable labeling sites is still important, not only for genotyping purposes but also for gene detection.

1.3. Common applications of OM in microbiology

The underlying concept of OM relies on the fact that a specific optical map corresponds to a particular nucleotide sequence. Based on this principle, OM has been applied in the areas of clinical and medical microbiology, including the epidemiology of foodborne microbial pathogens. Based on the same principle, OM has also been used as an auxiliary technique for whole-genome sequence analyses. Both applications are reviewed below (Box 3).

Comparative genomics and typing. Applications include microbial comparative analyses, such as genotyping and genetic recombination events. For microbial comparative analyses and/or typing, the principle of the technique is that the optical maps of different genomic fragments are characteristic of microorganisms of a given species, genetic lineage, or strain (19). Multiple examples are found in the liter-

ature regarding OM-based strain identification, characterization of pathogenic and antimicrobial resistant bacteria and outbreak investigations, or assessing genomic variations (Table 1). For instance, Johnson et al. (20) used OM to compare genomes and identify possible inversions in the *vanB* gene in vancomycin-resistant isolates of *Enterococcus faecium* isolated in a health care unit over a period of 11 years and all belonging to the same multilocus sequence type (MLST) clonal complex. Alexander et al. (21) used OM and DNA sequencing to identify the common ancestor of two travel-associated clinical isolates of *Escherichia coli* O104:H4 that shared the same MLST but presented distinct pulsed-field gel electrophoresis (PFGE) and antibiotic susceptibility profiles. The authors showed that horizontal gene transfer events were the major reason for the observed genotypic and phenotypic divergence (21). Similar conclusions were reached by Mellmann et al. (22) when comparing the *E. coli* O104:H4 isolate LB226692 responsible for the German outbreak of May 2011 with the hemolytic uremic syndrome isolate 01-09591 of 2001. Also working with *E. coli* isolates, Schwan et al. (23) used OM for rapid differentiation of uropathogenic strains while

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establishing subgroups of fluoroquinolone-resistant isolates.

The identification of large genomic rearrangements relevant to epidemiologic or evolutionary studies that researchers may not be possible to characterize with whole-genome sequencing is another useful application of OM. Genome rearrangements, such as insertions, deletions, translocations, and inversions, have been identified using OM in species as diverse as *Shigella flexneri*, *Salmonella enterica*, *Mycobacterium avium*, and *S. aureus* (24–28). The identification of specific clinically-relevant traits such as hemolysis, the presence of pathogenicity islands, virulence factor genes, or antibiotic resistance genes are also relevant OM applications since significant correlations have been observed between optical maps and those traits (23).

Genome assembly. Another common application of OM has been for whole-genome assembly. Here, the concept is that through the overlap of restriction enzyme fragment maps from OM, it is possible to assemble a complete genome (29). For example, OM proved to be valuable for the discovery of an inversion that, due to an assembly error, was not noted in the genome of *M. avium* strain M. ap K-10 (26). The company OpGen, Inc. developed a user-friendly technology that included all of the necessary steps to obtain, assemble into the whole genome, and compare optical restriction maps of an organism of interest, but the company has recently left the OM business. Ananthraman et al. (30,31) also reported new analytical methods and algorithms supporting mapping and assembly of randomly sheared genomic DNA molecules—specifically the Gentig algorithm. These were the developments underlying the above-mentioned initial shotgun OM maps constructed for *D. radiodurans* (2). The use of two or more restriction enzymes to generate independent optical maps for the same genome may provide additional information, increasing the accuracy of whole-genome assembly (32) (Table 1).

1.4. OM benchmarking

Over the past few decades, multiple techniques allowing the analysis of microbial genomes have become available. The major aims of these studies are as diverse as the elucidation of genome organization, determination of relationships between genotype and phenotype, and comparative genomics analysis for typing or evolutionary studies. Most of the time, the main approach relies

on endonuclease restriction maps, selective PCR amplification, or whole-genome DNA sequencing. The choice of method is based on multiple criteria, including user-friendliness, speed of performance, and portability of the output (e.g., nucleotide sequences made available in public databases), as well as its sensitivity and resolution. Often, a single approach is not able to provide the required information, and a combination of methods may be needed. The cost of the analysis must also be taken into account. Below, we address the application of OM as an alternative or as a complement to other techniques.

Comparison of OM and PFGE. The selection of methods used to identify microorganisms in a given sample depends primarily on the level of discrimination required. Low levels of discrimination may be sufficient to assess the presence of different taxonomic groups in a sample, but the discrimination between close relatives, such as pathogenic and innocuous variants of a given species, requires technical approaches with high resolution. The comparison of OM with PFGE is inevitable, as both methods involve the generation of large DNA fragments with restriction endonucleases that have a low cutting frequency. Over the past decades, PFGE became the standard technique used by public health agencies due to its accuracy and reproducibility between different laboratories; therefore, it has been considered the gold standard for microbial typing (33,34). However, current PFGE protocols are time-consuming, with lengthy restriction enzyme digests and extended electrophoresis times, and have low discriminatory power to distinguish bands of nearly identical size (34). OM and PFGE differ mainly in the fact that PFGE provides information about fragment

size, while OM provides information about both fragment size and location. Therefore, PFGE can sometimes be replaced or at least complemented by OM methods. Indeed, while PFGE may have too low a resolution to assess the relatedness of strains that show subtle differences (e.g., loss/gain/shift of 1 or 2 bands), OM can explain distinct band patterning (e.g., genomic inversion, insertion, or deletion) or even cases of great resemblance of PFGE patterns among unrelated strains. Given that the techniques rely on similar principles, OM and PFGE showed a similar resolution power in the discrimination of *S. enterica* Typhimurium isolates from the 2008–2009 Danish salmonellosis outbreak (28). In this study, isolates of the same cluster defined by multilocus variable number of tandem repeat analysis (MLVA), the primary method for *Salmonella* Typhimurium outbreak detection in Europe, were shown to be distinguishable by both PFGE profiling and OM.

OM as a complement to common genotyping methods. MLST, classically based on sequence analysis of seven house-keeping genes, has been used to establish phylogenetic relationships among bacterial strains of the same species (www.mlst.net/). This typing method generates highly portable results (i.e., data that can be shared among different users without loss of information or misinterpretation of the output) in the form of nucleotide sequences and has been implemented for clinically relevant bacteria and yeast as diverse as *Candida albicans*, *E. coli*, and *Helicobacter pylori*. Although MLST currently tends to be based on whole-genome analysis, due to reduced DNA sequencing costs, this typing method may still be laborious, time-consuming, and too expensive (35). The use of OM for the characterization of whole genomes of strains typically characterized by MLST is a valid approach since in some cases OM can have a higher resolution than MLST (21). In this respect, OM also has the capability to screen the accessory genome that is ignored by classical MLST approaches. However, ever-improving DNA sequencing and analysis tools may obviate the use of additional tools such as OM. DNA microarrays have also been used for genome-wide comparative studies and OM is also a useful complement to this approach (26). One of the advantages of OM is that, without DNA sequencing, it gives an unbiased overview of the whole-genome structure, allowing rapid detection of changes that may occur as a consequence

Box 2

For optical mapping (OM), DNA integrity must be maintained. Researchers should avoid extraction procedures that could shred DNA molecules (e.g., commercial kits, magnetic bead-based methods, agarose plug-based methods).

DNA restriction digestion and the analysis of the ordered restriction fragments by one of the labeling methods may improve the recognition of specific sequences and facilitate construction of the optical map, particularly when large DNA molecules (e.g. chromosomes) are analyzed. Rare-cutting restriction enzymes are frequently used. When DNA sequencing data from closely related strains are available, in silico OM restriction maps with the same enzyme can be obtained, allowing rapid comparison of the organisms (Table 1).

of recombination, deletions, or inversions, as well as the detection of repetitive regions (23).

OM for genome assembly. The reduction in cost of whole-genome sequencing has been a strong incentive for the direct genome analysis rather than investing in typing methods. Nonetheless, genome assembly and annotation are still time-consuming tasks that sometimes yields unreliable results. OM was demonstrated to be a useful tool for rapid, accurate, and reliable genome assembly, in particular to overcome the disadvantages of short-read technologies (e.g., Sanger, Illumina, or IonTorrent sequencing), and for the validation and/or correction of short-read assemblies containing duplications or inversions (36,37). A good example is the assembly of the genome of *Xenorhabdus nematophila* (Enterobacteriaceae family) (38) and the detection of an inversion in the *Xenorhabdus bovienii* genome (37). OM represents a useful, fast, and low-cost complement to whole-genome analysis, supporting reliable assemblies even in repetitive regions that are often difficult to assemble using conventional bioinformatics tools, avoiding extensive work on primer design and Sanger sequencing and, thus, facilitating comparative genomics approaches. Moreover, OM supports the

Box 3

Optical mapping (OM) is a cost-effective typing/identification method that allows the clustering of strains with similar traits. OM is also a powerful tool in comparative genomics for identifying genome rearrangements such as indels, inversions, and translocations in closely related strains. In addition, OM is an excellent complement for de novo whole-genome assembly. Because OM resolves highly repetitive DNA regions when coupled with short-read draft genome sequencing data, it saves time and expense by reducing or eliminating the need for Sanger sequencing to establish the order and orientation of the contigs. New applications include the simultaneous identification of genes combined with mapping. The combination of OM with cell sorting shows promise for the identification of pathogens in complex mixtures.

assembly of genomes from diploid organisms with multiple chromosomes (3,39). In spite of the continuous improvement of DNA sequencing techniques, in particular increases in read lengths, and of bioinformatics analyses, OM can still represent a valuable complementary approach to assembly of new and complex genomes, in particular highly dynamic genomes with large tandem duplications, pathogenicity islands, or antibiotic resistance gene cassettes.

2. OM in microbiology: A new perspective

One of the drawbacks of OM technology based on restriction maps is the inability to analyze small DNA molecules (<150 kb) since these molecules usually do not contain a sufficient number of restriction sites to perform a reliable alignment between the individual restriction maps. In recent years several approaches have been developed to increase the resolution of optical DNA maps (Figure 2). These new methods are based on sequence-specific labeling of DNA, without DNA restriction. This means that the labels can be placed at a higher frequency along the DNA molecule and that longer DNA molecules can be analyzed while still intact. Although these methodologies have so far not been used extensively for microbiology, there are good prospects for important applications in this field, as discussed below.

2.1. New methods of DNA processing for OM analysis

DNA Stretching. The two most common methods for stretching DNA for the new types of optical DNA mapping are either on a surface or by confinement in nanofluidic

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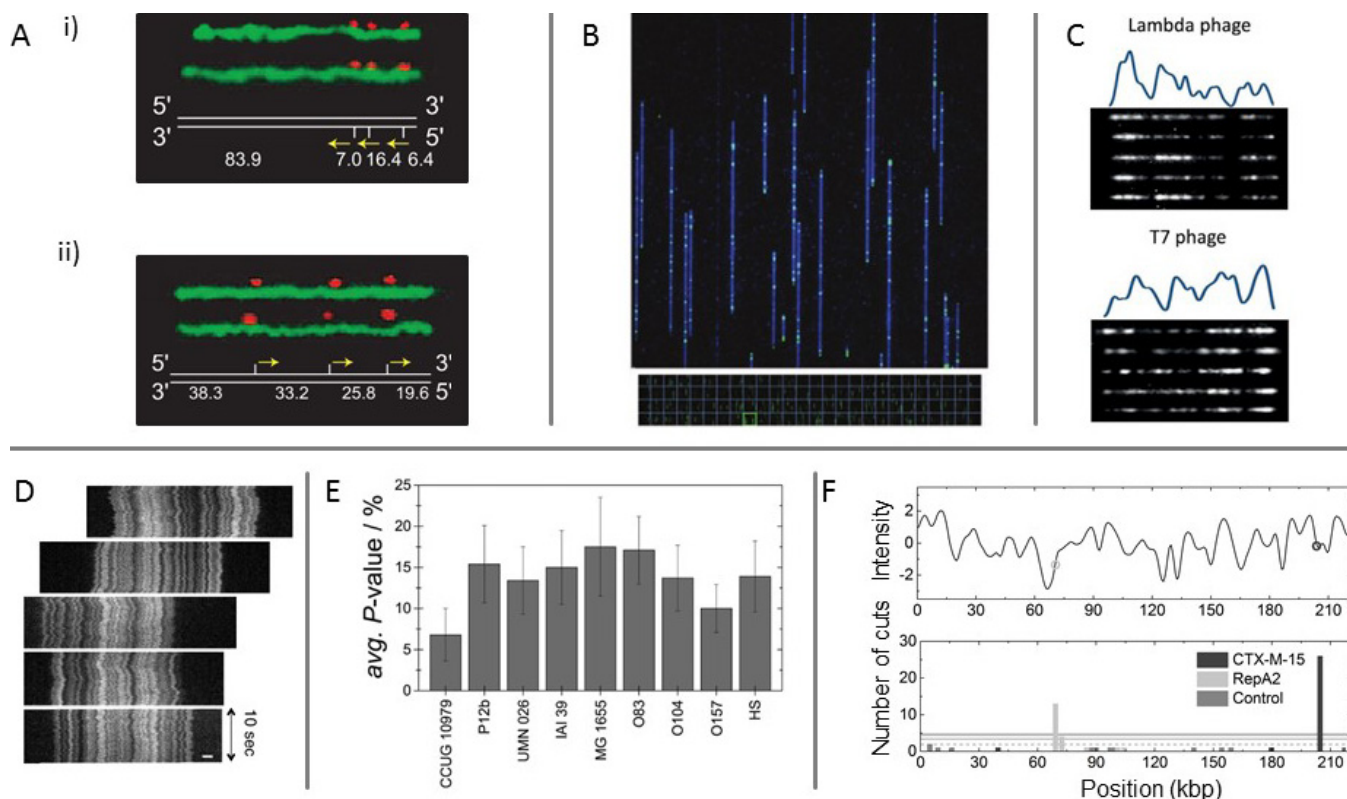


Figure 2. Experimental results from novel DNA optical mapping protocols. (A) Images of single nick-labeled bacterial artificial chromosome (BAC) DNA molecules (DNA backbones in green and FRET-imaged spots, corresponding to the nicking sites, in red) compared with patterns (in kb) predicted from the underlying DNA sequences for (i) BAC79 (113.7 kb) and (ii) BAC150 (116.8 kb). Adapted from Reference 48. (B) Fluorescence microscopy image of a $73 \times 73 \mu\text{m}^2$ field of view containing nick-labeled DNA molecules in parallel nanochannels. The high throughput is highlighted below, where several fields of view are shown. Reproduced by permission from Reference 49. (C) Amplitude modulation (AM) profiles of five single λ (upper panel) and T7 (lower panel) DNA molecules in nanofluidic channels after labeling with *M. TaqI* and AdoYnTAMRA. The molecules have been aligned to each other and the corresponding theoretical AM profiles. Reproduced with permission from Reference 53. (D) Raw kymographs of T4GT7 DNA obtained by denaturation mapping. The kymographs have been aligned to overlap the circularly permuted barcodes. Adapted from Reference 55. (E) Average *P*-values (error bars represent SE) when matching experimental barcodes for 12 DNA fragments to the full sequence of the correct strain (CCUG 10979) as well as 8 reference strains. Reproduced with permission from Reference 58. (F) Competitive binding-based theoretical barcode (top) for plasmid pUHH239.2 (pUHH) calculated from the DNA sequence. Rings mark the position of the *repA2* (light gray) and *bla*_{CTX-M-15} (dark gray) genes, respectively. Histogram (bottom) showing location of double-strand DNA breaks on individual plasmids treated with CRISPR/Cas9 loaded with a gRNA targeting either the *repA2* gene (light gray) or the *bla*_{CTX-M-15} gene (dark gray), as well as a control linearized by light (gray). The vast majority of the double-strand breaks appear at the predicted locations when using Cas9, confirming that the genes are present. Adapted from Reference 60.

channels. In the first method, a droplet of DNA solution is deposited on a positively charged surface (often a glass slide treated with polylysine) or a hydrophobic surface and then stretched between the surface and a coverslip by the receding meniscus of the droplet (40–42). Alternatively, stretching DNA by confinement in nanofluidic channels offers a uniform stretching with clear advantages for analysis and visualization of the optical map (43). When compared with surface stretching, nanofluidics is more laborious since manufacturing of nanofluidic chips requires state-of-the-art lithography techniques (44). Stretching in nanochannels is, however, more homogeneous since it is done in solution. Stretching of single megabase DNA molecules in nanoscale structures has been demonstrated, opening up the possibility of studying genetic arrangements on ultralong length scales (45–47). An alternative that is much simpler to fabricate

and use is based on nanoslits. The stretching of the DNA is enhanced by working under low-ionic-strength conditions (48) or allowing the DNA to straddle the nanoslits (45,46). In the latter case, the two ends of the DNA extend outside the nanoslit, forming a dumbbell geometry. The entropy difference between the DNA residing outside and inside the nanoslit results in tension stretching of the DNA. The stretching of the DNA is enhanced by working under low-ionic-strength conditions (48) or allowing the DNA to straddle the nanoslits (45,46). The presence of DNA dumbbells contributes entropic tension that stretches out the DNA. Overall, in contrast to surface-based methods, nanofluidics has greater potential to be used for fully integrated devices, as discussed below.

DNA Labeling. Two major DNA labeling methods have been used in modern OM applications: enzymatic labeling and affinity-based labeling. Enzymatic labeling schemes

use a DNA-nicking enzyme that recognizes a specific sequence and promotes the addition of a labeled fluorophore. The method of nick labeling was introduced for OM applications by Jo et al. (48) (Figure 2A). In brief, a nicking enzyme with a recognition site of typically 4–7 bases is utilized to create site-specific, single-strand nicks in the DNA that are subsequently filled with fluorescently labeled nucleotides using a DNA polymerase. The DNA molecules are then stretched, and the individual fluorescent dots are located. The labeled sequence motifs are characteristic of a given DNA molecule, and matching of labeled DNA pieces in different DNA molecules can provide maps of entire genomes. This principle is used in the commercially available optical mapping benchtop machine provided by BioNanoGenomics (San Diego, CA) and has been used in recent studies on human DNA (49–51) (Figure 2B).

Another enzymatic labeling process uses methyltransferases to label specific sequence motifs. Some methyltransferases are able to transfer larger substrates than methyl groups, and this property can be used to introduce fluorophores into specific DNA sequences (52). Since this type of labeling does not damage the DNA, it is possible to label the DNA densely and thus obtain high resolution. In an interesting application of this method, Grünwald et al. (53) were able to type and identify bacteriophages based on an amplitude modulation profile in which the fluorescence intensity along the DNA molecules stretched over nanofluidic channels exhibited a unique molecular fingerprint (Figure 2C).

One limitation in using nicking enzymes is that the length of the specific target site of the enzyme (4–7 bases) is too short to identify specific genome regions. To overcome this hurdle, McCaffrey et al. (54) used a nicking enzyme based on the CRISPR/Cas9 system. While the wild-type Cas9 enzyme cuts both strands of the DNA molecule, the commercially available mutant enzyme Cas9 D10A, lacking one of the nuclease activity subunits, causes a nick in only one of the DNA strands, instead of breaking the

molecule. After the nicking, the procedure is identical to the other nick-labeling schemes. Since the recognition site of Cas9 D10A is ~20 base pairs, the specificity compared with other nicking enzymes is improved. In fact, a recognition site of 20 base pairs means that a specific gene can be identified and targeted, even in the human genome. McCaffrey et al. (54) used this principle to label several different genes that are not accessible with traditional nick labeling.

In an alternative to enzymatic labeling, DNA can be sequence-selectively labeled based on non-covalent interactions. An example of this principle is the denaturation mapping technique developed by Reisner and coworkers in 2010 (55). This method relies on the fact that adenine-thymine base pairs (AT), which have two hydrogen bonds, melt at slightly lower temperatures than guanine and cytosine base pairs (GC), which have three hydrogen bonds. This labeling method uses DNA intercalating dyes that require double-stranded DNA, commonly the dimeric cyanine dye YOYO. Therefore, the fluorescence will vanish in melted regions. By controlling the temperature, it is possible to tune the staining to target DNA regions with a given minimum

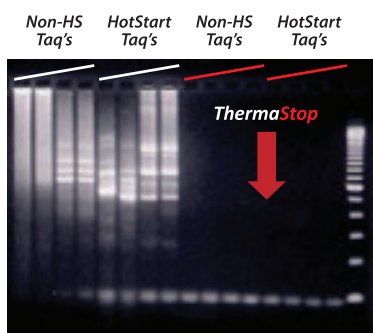
GC content. As a result, the labeled DNA molecule shows weak fluorescence emission in AT-rich regions and a strong emission in GC-rich regions (Figure 2D). The usefulness of denaturation mapping was demonstrated on various model DNA sequences, including viral DNA, bacterial artificial chromosomes (BACs), and even DNA extracted from the yeast *Saccharomyces cerevisiae*, in which it was possible to generate 360-kb long denaturation maps (56). This procedure offered the opportunity to screen >50% of the 12.1 Mb of the yeast genome based on the comparison of the optical maps from 84 DNA fragments with the computationally predicted maps.

As an alternative to denaturation mapping of DNA, Nyberg et al. (57) demonstrated that a sequence-selective ligand could be used to prevent YOYO from binding to DNA. Instead of controlling temperature to achieve desired denaturation maps, this process relies on the promotion of competitive binding where AT-rich regions are blocked from YOYO binding by the non-fluorescent polyamide netropsin. This molecule has high affinity for quadruples of AT base pairs and will leave the GC-rich regions free to bind the fluorescent dye YOYO. The result

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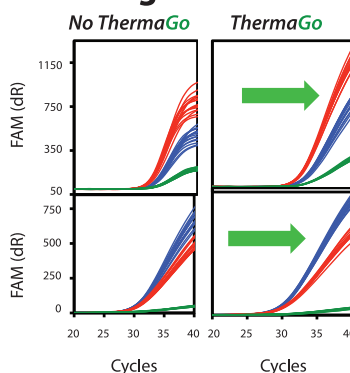
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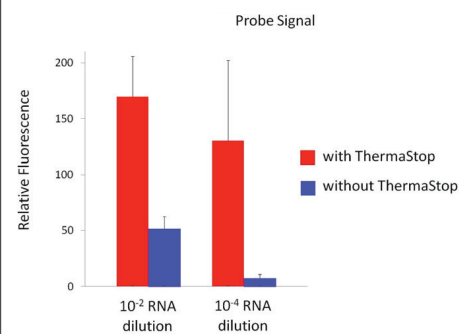
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is a reproducible fluorescence intensity variation along the stretched DNA molecule that corresponds to alternating GC-rich and AT-rich regions, characteristic for each DNA molecule. This labeling method was applied to identify a given strain of *E. coli* in a library of nine strains. The approach was based on fitting experimental barcodes of large pieces (50–160 kb) of DNA to the theoretical barcodes of all of the full *E. coli* genomes available in the library (Figure 2E) (58).

Bacterial Plasmids. The competitive binding assay, using netropsin and YOYO, has also been applied to the characterization of bacterial plasmids. Since plasmids are small circular DNA molecules, they can be distinguished from linear fragments of chromosomal DNA (59). In a recent application, Nyberg et al. (16) compared experimental barcodes obtained for 3 plasmids with different sizes [RP1 (60.1 kb), R100 (94.3 kb), and pUUH239.2 (220.8 kb)] with the theoretical barcodes. The good agreement between the experimental and theoretical barcodes observed for these plasmids enabled the identification of the plasmids R100 and pUUH239.2 in a theoretical barcode database based on 3192 plasmids >20 kb (NCBI RefSeq; www.ncbi.nlm.nih.gov/refseq/). These studies led to the conclusion that this OM approach should enable the identification of plasmids larger than ~70 kb, while for plasmids with sizes ranging 30–70 kb, the reliability of identification may depend on the uniqueness of the respective barcode. In the investigation of an outbreak at a neonatal ward, OM was used to identify plasmids in distinct bacterial strains and species hosted by different patients (15). The authors concluded that the underlying cause of the polyclonal outbreak was a single plasmid that was common to all clinical samples and probably had been propagated by horizontal gene transfer.

A limitation of OM for plasmid characterization has been identification of acquired antibiotic resistance or virulence genes, which due to their small size barely yield a distinct signal in the optical maps. Such a limitation can be overcome thanks to the recently described labeling system based on the CRISPR/Cas9 system (60). The application of this system to plasmids has the advantage of simultaneously identifying a target gene and generating a linear molecule. Since the cut will occur in a specific site, the barcode will be identical in all identical plasmids. If, on the other hand, the plasmids are linearized by other means, the barcodes

will be circularly permuted and the identity between different plasmids will not be obvious. OM can thus, in a single experiment, allow the determination of different characteristics for a plasmid sample (e.g., the number of different plasmids, their size, and the presence of a specific gene, as well as the barcode of each plasmid) that will allow further epidemiological surveys (Figure 2F).

2.2. Future directions— Integration and automation

An ideal tool for diagnostics is based on a self-sufficient platform that is easy to use and highly cost-effective. To build a comprehensive lab-on-a-chip device, several components need to be present: extraction and enrichment of the cells of interest, lysis of cells, DNA extraction, labeling of the DNA, and introduction of the DNA into nanochannels for direct visualization.

Fast and efficient cell sorting is essential for successful DNA-based diagnostics in order to select and enrich the cells of interest. While capture efficiency may be important for rare cells, in OM, purity is crucial so that the DNA of interest is not overwhelmed by background DNA, for example from white blood cells. To simplify the sorting and make it amenable to integration with nanofluidics for the DNA analysis, standard immunolabeling and subsequent cell sorting using, for example, fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS) is too complex. Instead, microfluidic label-free sorting schemes based on, for example, acoustophoresis (61), inertial focusing combined with Dean flow (62), or deterministic lateral displacement (DLD) (63) are preferred.

Once the cells of interest are collected and purified, they need to be lysed to enable DNA extraction. On-chip cell lysis may mimic standard procedures, or it may take advantage of the specific capabilities afforded by microfluidics. One example is based on the treatment of bacteria to form spheroplasts that are subjected to subsequent osmotic shock (64). To clean up the DNA from the lysis, DLD can be used to move the DNA from the lysis buffer across flow stream lines to fresh buffer (65). This approach can also be used to clean up DNA after staining. Here, dielectrophoresis (DEP) can be useful to trap the DNA while other components are rinsed away. Shaping the field by dielectric obstacles makes it possible to trap the DNA without any integrated electrodes (66). One important challenge in

the transfer of the DNA to the nanochannel is entanglement of the DNA. This can be remedied by post arrays positioned at the entrance of the nanochannels that acts as combs (67). A fully integrated lab-on-a-chip has not yet been realized, and it is necessary to consider the difficulties in processing the sample flow through the device. As a first step, it may be worthwhile to automate parts of the process and perform other steps in bulk.

3. General considerations

OM provides a coarse genome-wide scaffold, an important contrast with the paired-end data generated by DNA sequencing, which frequently results in fragmented scaffolds (19). Therefore, OM can be regarded as a good complementary methodology to other genome analysis techniques. Until now, one of the most important applications of OM has been genome assembly, with validation of the assembly provided by bioinformatics methods (68). Another application has been for microbial typing, mainly from an epidemiological perspective (68). Nonetheless, OM can be used in the genomic characterization of any type of microorganism.

Among the advantages of OM for typing is its speed. An optical map can, with recently developed OM protocols, be constructed within as little as 24 h after receiving a DNA sample. Optical maps are therefore an attractive alternative to sequencing methods, as the construction of a paired-end library can take days. Note however that it may be beneficial to combine OM data and paired-end data due to their complementary characteristics (19).

Another advantage of OM is cost-effectiveness. Novel OM techniques use commercially available reagents in combination with epifluorescence microscopy, standard equipment, and reagents found in many microbiology laboratories. Furthermore, with emerging developments in plastic nanofluidic devices, it has been suggested that the cost per device could be kept as low as \$3 (69). The small amount of sample needed for OM is another factor contributing to work-time reduction, as bacterial cultivation steps can potentially be shortened or even omitted. The short time needed for OM analysis is particularly relevant for epidemiology. A faster diagnosis means that patients can receive the correct treatment earlier and that the further spread of antimicrobial resistance can be hindered (15,16). The reduction

of manipulation through the automation of OM would eventually allow the miniaturization of the process, with expected increases in throughput as well as reductions in cost (1).

The recognized advantages of OM-based methods have encouraged recent developments in DNA stretching and labeling methods. The simplicity of sample preparation and analysis, combined with the increasing specificity that has been achieved for this technique, places OM in a unique position for the development of integrated and miniaturized diagnostic systems.

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Competing interests

The authors declare no competing interests.

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