Advances in the analysis of DNA sequence variations using oligonucleotide microchip technology

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The analysis of DNA variation (polymorphisms and mutations) on a genome-wide scale is becoming both increasingly important and technically challenging. An integration of a growing number of molecular biological methods of DNA-sequence analysis with the high-throughput feature of oligonucleotide microarray-based technologies is one of the most promising current directions of research and development.

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Current Opinion in Biotechnology 2001, 12:53–58

0958-1669/01/$ – see front matter

Abbreviations
PCR polymerase chain reaction
SDA strand-displacement amplification
SNP single nucleotide polymorphism

Introduction

Oligonucleotide microarrays, or DNA chips, are miniature parallel analytical devices containing libraries of oligonucleotides robotically spotted (printed) or synthesised *in situ* on solid supports (glass, coated glass, silicon or plastic) in a way such that the identity of each oligonucleotide is defined by its location. DNA-chip technologies are distinguished by the sizes of arrayed DNA fragments, the methods of arraying, the chemistries and linkers for attaching DNA to the chip, and the hybridisation and detection methods [1–3]. Although the chip manufacturing, data collection and processing are very important aspects of DNA-chip technology, this review mainly focuses on the integration of different molecular biological methods of sequence analysis with the multiplexing nature of microarrays.

Microarray-based technology for analysing the expression levels of thousands of genes per single experiment is well established [1,2,4,5]. Another application, the analysis of point mutations and single nucleotide polymorphisms (SNPs) in the genomic DNA of different organisms, is more complicated and currently under intensive development. SNPs, common variations among the DNA of human individuals, are being uncovered and assembled into large SNP databases that promise to enable the dissection of the genetic essence of disease and drug response [6–10]. The large number of SNPs provides a rich set of markers that can be used in a wide variety of genetic studies. The identification of a complex set of genes that cause a disease will require both linkage and association analyses of thousands of polymorphisms across the human genome, in thousands of individuals. There is an urgent need for a fast, specific, sensitive, reliable and cost-effective method for the genome-wide polymorphism analysis. With the aid of DNA microarrays, numerous sequences can be investigated, in parallel, by confining individual analyses to specified areas of the chip through hybridisation with immobilised oligonucleotides. Microarray-based methods are strong competitors to conventional methods that dominated throughout the 1990s, gel-based fluorescent DNA sequencing and other gel-based methods of sequence variation analysis [11•]. The performance of numerous parallel analyses on the small surface of oligonucleotide microarrays is one of the most promising approaches for large-scale SNP genotyping.

This review outlines current progress in the integration of SNP- and mutation-detection methods with oligonucleotide-array technologies. For recent overviews of related topics see references [12–14].

The hybridisation of complementary strands of DNA is the underlying principle of all methods of chip sequence analysis. The direct hybridisation of a fluorescent-labelled derivative of analysed DNA or RNA (the target) to chip-bound complementary allele-specific oligonucleotides (the probes) has been applied for genomic analyses focused on the identification of specific sequences, mutations, analysis of SNPs and the detection of changes in gene expression. The target preparation and the fidelity of hybridisation are two major concerns of this type of assay.

Multiplex polymerase chain reactions and other target DNA amplification methods

DNA regions spanning the genomic mutations or SNPs usually should be amplified before, or in the course of, the analysis. Thousands of parallel polymerase chain reactions (PCRs) should be performed for each human genomic DNA sample in order to carry out a large-scale analysis. To avoid this complicated and expensive procedure, a developing of technology for combining many individual PCR reactions per tube (multiplexing) was suggested [7,15,16•,17•]; however, currently available technologies of multiplex amplification are limited in that only a small number of different DNA regions can be simultaneously amplified. Many of the problems associated with multiplex amplification are the result of non-specific interactions between primers. Much emphasis, therefore, has been placed on the reduction of primer–primer interactions through the use of specialised primer designs, blocking oligonucleotides, and the optimisation of primer–reagent concentrations [15,18–22].

Another way of decreasing primer–primer interactions is to anchor or physically separate amplification primer-pairs from one another. A process where PCR primers are covalently coupled to latex beads is described as 'Bridge Solid Phase DNA amplification' [23]. The efficiency of the PCR amplification is limited in the case of both anchored primers owing to the physical constrains of the amplified double-stranded DNA tethered to the support through both ends. A release of the anchored amplicon and one (or both) primer(s) should significantly improve the amplification (see below).

The anchored DNA amplification could also be done using strand-displacement amplification (SDA). SDA uses the combined effects of a restriction endonuclease and DNA polymerase, and does not require temperature cycling [24]. The SDA methodology has been combined with microelectronic chip technology [25,26•,27••] by anchoring SDA primers onto the chip in discrete locations, electronically hybridising templates and performing SDA, *in situ*, on the chip [27••]. Complete strand-displacement of single-stranded amplicons occurs with each cycle of replication in SDA; however, because the single-stranded copies are unconstrained, they are also free to drift to other areas of microchip array. This may limit the sensitivity and specificity of the anchored SDA.

Nucleic acid sequence-based amplification is another isothermal amplification method, which may in some cases offer advantages over PCR. This method typically uses RNA as a template and includes cycles of three successive enzymatic reactions with reverse transcriptase, RNase H and T7 RNA polymerase producing single-stranded antisense RNA as the major amplification product [28,29].

Pre-formed circular oligonucleotides can be used as universal amplification templates in rolling-cycle amplification to quantify hybridisation/ligation events on surfaces that contain site-addressable probes [30]. Following the allele-specific ligation, the resulting glass surface-immobilised oligonucleotide probe can be hybridised with the complementary circular DNA and extended by DNA polymerase. This approach generates a strand that represents multiple head-to-tail copies of the DNA sequence and has been shown to be both sufficiently specific and sensitive to detect single-copy sequences in human genomic DNA. An obvious limitation of this method is the very low amount of target DNA at the initial hybridisation and ligation steps of the assay.

A radical way to avoid the problems of target amplification would be to analyse genomic DNA sequences directly, using different hypersensitive detection techniques such as branched DNA or dendrimers, surface-enhanced resonance Raman scattering and fluorescence-correlation spectroscopy [13]. These techniques, however, cannot be readily applied to the analysis of human genomic DNA.

Target DNA labelling

Another drawback of typical target preparation for the hybridisation type of assay is the need for an additional enzymatic reaction to incorporate biotinylated or fluorescent-labelled conjugated nucleotide analogs for the labelling of the target. It is not only time consuming, and rather expensive, but can also change the levels of targets originally present in the sample. The use of such a fluorescent technique as molecular beacons [31] allows one to work with unlabelled targets. Molecular beacons are oligonucleotide probes with a hairpin-shaped structure in which the 5′ and 3′ ends are self-complementary, bringing a fluorophore and a quencher into close proximity. Fluorescence is restored when the molecular beacon binds to a complementary target nucleic acid. Recently, surface-immobilised molecular beacons were used for screening of unlabelled-DNA targets with randomly ordered fiber-optic gene array [32•].

Many new approaches of sequence analysis include additional processing of the initial duplex between the oligonucleotide probe and the target molecule on the chip (see below). It is often convenient to perform the labelling in parallel with this additional treatment.

Fidelity of hybridisation

Differential hybridisation with allele-specific oligonucleotide probes is the most commonly used procedure in the microarray format. The specificity of genotyping by hybridisation depends strongly on the nucleotide sequence context of the SNPs and the hybridisation conditions. The use of *in situ* synthesised high-density probe arrays influences hybridisation specificity because of the presence of truncated probes, target-DNA binding to multiple probes and probe–probe interactions [33]. These problems can be alleviated by fragmentation of the target, by building vast redundancy into the array and by carefully selecting oligonucleotide sequences [4,17•]. Other proposed modifications to distinguish heterozygous from homozygous alleles on the oligonucleotide array include the use of two-colour fluorescence analysis [34], incorporation of nucleotide analogues in the array sequence [35], the use of adjacent co-hybridised oligonucleotides [36], the monitoring of melting curves of the hybrids [37] and an electronic stringency changer [26•]. The power of genotyping SNPs using high-density allele-specific oligonucleotide arrays was shown to be limited in recent mapping studies in which 400–500 SNPs were analysed and the correct genotype could only be assigned at 60–70% of the analysed sites [7,38•].

Specially designed 'bar code', 'tag', 'zip-code' or 'cZipCode' arrays consist of selected oligonucleotides with similar hybridisation characteristics and minimal cross-hybridisation. Sequences with possible secondary structure are eliminated. This kind of array is universal because the sequences, complementary to immobilised probes, could be appended to any set of specific primers in the course of target-DNA processing [16•,39,40••,41].

Enzymatic reactions for better discrimination

A popular development in array technology is the use of enzymatic reactions on the oligonucleotide arrays to enhance the discriminating power of the hybridisation.

Single-base primer extension is one of the most popular methods for high-throughput genotyping using SNPs. DNA polymerase uses a tethered oligonucleotide as a primer in an allele-specific extension of primer–template complexes with labelled dideoxy-nucleotide triphosphate terminators. This reaction requires the perfect base-pairing adjacent to the incorporation site at the 3′ end of the primer. The enzyme incorporates and extends only one terminating base – that which is complementary to the next base in the target. This process, known as minisequencing [42,43•], genetic bit analysis [44] or arrayed primer extension [45•], was successfully adapted to the microarray format. Compared with hybridisation with allele-specific probes in the same format, minisequencing showed one order of magnitude higher power of discrimination between homozygous and heterozygous genotypes [42].

Dubiley *et al.* [43•] have shown that the allele-specific single-base extension method, or multiprimer assay, gives discrimination results similar to those achieved by minisequencing or multibase assay. In the allele-specific extension method, only a single detection reaction with a single fluorophore is required per sample in contrast to four fluorophores or four detection reactions with the minisequencing method. Both reactions are carried out at an elevated temperature to amplify the extension signal, owing to the fact that one molecule of the target DNA could successively hybridise and catalyse the extension of several probes.

Pirrung *et al.* [45•] used a nested set of primers for each individual site within a template. Each single-nucleotide site in the template is involved in extensions with multiple primers, giving high redundancy in the data collection. Unknown mutations in a known sequence (deletions, transversions and up to two base-insertions) can be readily found, based on the arrayed primer extension reactions on both strands.

Another type of primer extension method, the allele-specific amplification [46], relies on the ability of Taq DNA polymerase to extend a primer hybridised to a DNA template, where the 3′ base of the primer matches its complementary base on the template. Recently, this method was combined with direct on-chip PCR amplification to detect drug-resistant mutations (SNPs) in *Mycobacterium tuberculosis* genomic DNA [47••].

Recently, Pastinen *et al.* [48••] have described a new method that relies on the sequence-specific polynucleotide extension, by a reverse transcriptase enzyme, of immobilised allele-specific primers that differ at their 3′-nucleotide defining the alleles. In the assay, one or two fluorescent nucleotide analogs are used to label extending primers in the course of the extension reaction. To prepare target DNA, multiplex PCRs yielding amplicons tailed with a 5′- T7 RNA polymerase promoter sequence are performed. The PCR products are then added directly to the primer array, along with the reaction mixture that contains both T7 RNA polymerase and rNTPs, in order to generate RNA targets from the PCR products, and reverse transcriptase and labelled dNTPs for the actual allele-specific genotyping reaction. The reverse transcriptase-assisted reaction provides highly specific allele distinction, as evidenced by its ability to detect minority sequence variants present in 5% of a sample at multiple sites.

Methods that relate to primer extension and use ligase have been developed [22,49] and are being adapted for use with arrays as multiplex oligonucleotide-ligation assays [40••,50,51,52••]. Barany and co-workers [22,40••,52••] have developed a very sensitive microarray method for multiplex detection of low abundance point mutation. This method combines multiplex PCR and the multiplex ligase-detection reaction with ‘zip-code’ hybridisation. Thermostable DNA-ligase links two adjacent oligonucleotides annealed to a complementary target if the nucleotides are perfectly base-paired at a junction. The assay allows for accurate detection of single-base mutations presented at 1% or even less of the wild-type sequence. Ligase-detection reaction is better than PCR for multiplexing, since several primer sets can ligate without the interference encountered in polymerase-based assays [22,40••,52••].

In addition to those mentioned above, a variety of new technologies are available. These technologies include invasive cleavage of oligonucleotide probes, mass spectroscopy, fluorescence polarisation and fluorescence resonance-energy transfer-based techniques, and all have the potential to be combined with microarrays.

Integration of *in situ* PCR amplification and SNP analysis on microchip containing gel-immobilized oligonucleotides

Recently, a new oligonucleotide array-based technology has been developed in our group (S Tillib, B Strizhkov, A Mirzabekov, unpublished data). This technology allows for multiple, independent amplifications followed by detection processes anchored at specific addresses on the microchip. MAGIC Chip™ (MicroArray of Gel-Immobilized Compounds on a Chip) with gel pads created on a glass surface [36,37,43•,47••] is a convenient platform for the assay. The method is based on the successive use of different enzymatic reactions and has several key features. First, specifically designed primers are initially anchored (immobilised) within gel pads of 0.4 nanolitres in volume. A set of primers for each gel pad is chosen to perform an analysis of a particular genetic locus. The DNA to be amplified is enriched at the gel pads by its hybridisation with immobilised primers. Second, mineral oil is used to fully separate wet gel-pads after the initial hybridisation step. The separated gel pads of the microchip are used to simultaneously perform specific parallel analyses within each of many gel pads (nanolitre test tubes). Third, one or two amplification primers are enzymatically detached from the gel matrix (within the gel pads surrounded with oil) and the PCR

amplification is performed under oil. Fourth, inner permanently immobilised inactive primer is used for anchoring the amplified DNA within the gel pad after oil is replaced with water solution. Then, inactive primer is made active by the enzymatic removal of a 3′-blocking group and allele-specific primer extension of the activated inner immobilised primer, hybridised with DNA, is carried out. The final analysis of the extended immobilised primers involves their hybridisation with fluorescently labelled oligonucleotide probes. The method was applied to simultaneously identify several abundant drug-resistant mutations in three genes of *M. tuberculosis* by using approximately 10^5 copies of genomic DNA (S Tillib, B Strizhkov, A Mirzabekov, unpublished data).

The described method of parallel amplification and analysis of multiple DNA sequences directly using an oligonucleotide microchip illustrates the high potency of the combination of a high-throughput array-based microchip technology and efficient enzymatic reactions for DNA analysis. We believe that single-base extension and other methods of SNP analysis could be used directly on the array-based microchip in conjunction with the described combinatorial PCR procedure. The described procedure could be easily modified for a particular analysis. The first part of it, amplification, is completely separated from the following detection step. Additional substrates, oligonucleotides, and enzymes could be used after the first PCR step and the hybridisation of amplified target-DNA with immobilised primer.

Conclusion

Described above are methods of sequence-variation analysis using oligonucleotide microarrays that are still in the developmental stage. Until now, no single technology, array format or chemistry has gained wide acceptance as the technology of choice. Nevertheless, microarray-based methods, with their vast diversity, are clearly becoming the methods of choice and promise to revolutionise sequence analysis in the near future. The efficient selection of oligonucleotides, the use of electronically enhanced hybridisation, the integration of effective enzymatic methods of amplification and discriminatory hybridisation on an oligonucleotide microchip, the development of highly integrated microchip-based procedures of DNA isolation, amplification and analysis are highly promising directions for future development.

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