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COMMUNICATION

DNA chips: a new tool for genetic analysis and diagnostics

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Summary – DNA chips are miniaturized microsystems based on the ability of DNA to spontaneously find and bind its complementary sequence in a highly specific and reversible manner, known as hybridization. Labeled DNA molecules in a sample are analyzed by DNA probes tethered at distinct sites on a solid support. The composition of the DNA sample is then deduced by analyzing the signal generated by labels present at each probe site. Applications are widespread: fundamental research, cancer or microbiology diagnostics, genotyping, gene expression, pharmacogenomics, and environmental control. Medical application consists, for example, in the identification and detection of mutations in genes responsible for cancers, or DNA chip analysis of individual polymorphisms which may provide a guide towards the most efficient treatment. In the environmental and agro-industrial fields, DNA chips show great promise in rapidly testing microorganism content, contamination or pathogenicity. DNA chip dimensions offer hybridization sites in the 50-200 micron range, producing arrays ranging from 100 to 1 000 000 different probes per cm^2. © 2001 Éditions scientifiques et médicales Elsevier SAS

DNA chip / gene analysis / genomics / microarray / mutation

The applications of DNA chip analysis are widespread and gaining ground: fundamental research, human genetics, infectious disease diagnosis, genotyping, gene expression monitoring, pharmacogenomics, environmental control. Recent articles have described the identification and detection of mutations in genes responsible for cancers, or how DNA chip analysis of individual polymorphisms may guide the clinician towards the most efficient treatment for his patient's particular form of disease. In the environmental and agro-industrial fields, DNA chips show great promise in rapidly and extensively testing microorganism content, contamination and pathogenicity. Access to DNA chip technology has become a technical and economic priority for academic and industrial institutions, and is estimated to reach a 650 million dollar market in the next five years.

'Labs-on-a-chip' are also miniaturized microsystems but carry out a higher number of analytical steps on a biological sample. They include channels, fluidics and thermal zones to bring about various enzymatic reactions in order to amplify, purify, and label biological samples. Lab-on-a-chip and DNA chips are complementary parts of a miniaturized analysis and are currently mainly used in conjunction with fluorescent labeling. A laser-based read-out system is commonly used to determine and eventually quantify the hybridized sequences.

Although hybridization techniques for analyzing DNA have been known to molecular biologists for

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decades, the keen interest that is now being shown in these new technologies has stemmed from a number of key innovations:
– the use of a planar solid support such as glass or silicon, facilitating miniaturization and high-density analysis;
– powerful high-resolution detection offered by fluorescence;
– the development of high-density spatial synthesis of short DNA probes (oligonucleotides) by photolithography or ink-jet printer technology. DNA chips dimensions offer hybridization sites in the 50–200 micron range, producing arrays from 100 to over 10 000 or even 400 000 different probes on cm^2 areas.

MANUFACTURING DNA CHIPS

DNA chip manufacturing is achieved either by 'on-chip' technologies where oligonucleotide probes are chemically synthesized in situ, or by 'off-chip' technologies where DNA probes are previously synthesized, purified and controlled before being grafted onto the substrate using a number of technologies such as mechanical, ink-jet or electrochemical deposition [1].

Initially, this type of technique was used on nylon or nitrocellulose membranes [2] with radioactive detection. Then, in order to increase the number and density of fixed probes and to be compatible with fluorescent detection, other supports have emerged such as glass [3], polypropylene sheets, polyacrylamide gel pads [4] and silicon [5]. The precise location of probes can be reached by photochemistry [6] or through micromechanical devices. The complexity of the array is adapted to the application requirements *(figure 1)*.

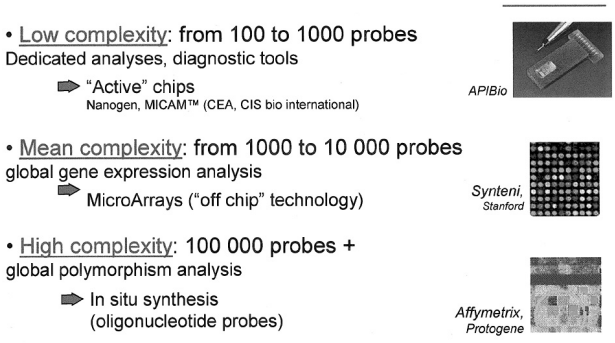


Figure 1. Different DNA chips for different technology.

Affymetrix, founded in 1992, uses photolithographic masking borrowed from the semiconductor industry and is clearly the current leader in the chip manufacturing industry. This company has signed collaborative contracts with most large pharmaceutical groups such as Glaxo Wellcome, Pfizer, Aventis, Novartis, bioMérieux, etc.

However, this leadership is now being challenged by a growing number of companies: HySeq, Incyte, Nanogen, Caliper, etc. With a strong foothold in microsystem development, Motorola has also joined the race. Among these, only a few European companies are present (BioRobotics in the United Kingdom, Clondiag, BioChip Technologies in Germany). In France, the French Atomic Energy Commission (CEA) has officially launched its 'BioChip project' based on its proprietary electrochemical deposition technology developed early in 1993 [7], and is contributing to the foundation of a new company whose activity is focused on chip manufacturing. These new actors are confident that they will be able to play a significant role in the coming years, as the market needs more flexible and cheaper technologies.

As in situ synthesis remains an attractive method for manufacturing high-complexity chips with hundreds of thousands of probes (without having to worry about handling and storing each probe), other companies and research laboratories have entered the race by proposing lower-cost or higher-yield alternatives (without photomasks). Protogene uses piezoelectric pipetting to successively deposit the four base precursors to build the DNA molecule in situ on a hydrophilic/hydrophobic 2-D structured surface. The University of Wisconsin proposes a Maskless Array Synthesizer whereby photolithography is not carried out using masks but via digital light processor technology from Texas Instruments. This technology is based on 480 000 tiny aluminum mirrors arranged on a computer chip.

'Off-chip' technologies have attracted interest, as probes are synthesized beforehand (with better yields), and quantified and purified before being grafted onto the substrate. Some technologies use contactless techniques *(figure 2)*. The result is a better control over probe quality and quantity, wide versatility in the choice of supports and a higher confidence in the chip readout. Changing single bases or whole probes on an array involves little expense other than synthesizing a new DNA molecule, and allows a more flexible update of products.

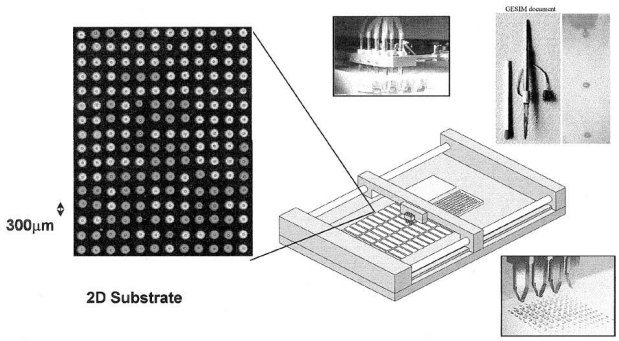


Figure 2. Piezoelectric technology.

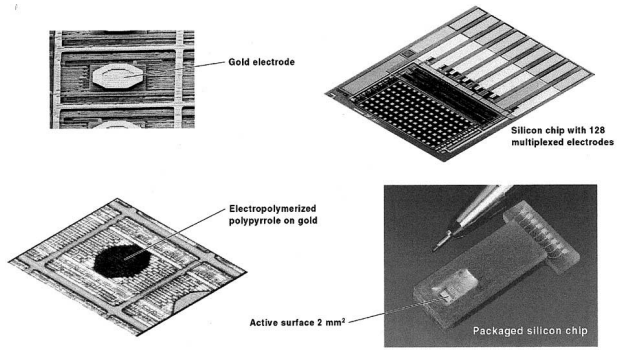


Figure 3. The MICAM™ technology.

In 'off-chip' technologies, the nature of the probe is more flexible as well. Depending on the type of application, longer probes (typically over a 100 bases long), PCR products, or PNAs may be grafted onto the substrate. In developments aimed at the protein world, some technologies have proven to accommodate peptides, antibodies or even enzymes.

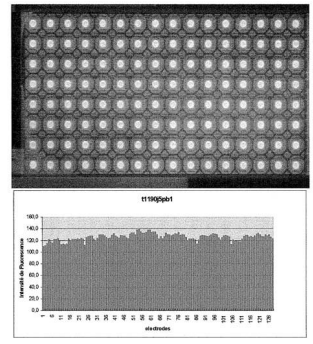
Other alternative technologies include using silicon substrates instead of glass (Nanogen, CEA). Microelectronic components are integrated into the silicon base, e.g., multiplexed circuits for active addressing of oligonucleotide probes to electrodes (CEA); or they produce electrical fields which enhance on-chip hybridization and stringency dynamics (Nanogen). These substrates may also integrate thermal control elements, microvalves, and microfluidic devices.

MICAM™ CHIP TECHNOLOGY

At CEA, first-generation DNA chips have 128 gold sites on a multiplexed silicon substrate on which oligonucleotides are fixed using pyrrole linkers in an electrochemical process. The process is named MICAM™ (MICrosystem for Analysis in Medicine). The construction of the MICAM™ DNA chip is based on the fixation of 5'pyrrole-labeled oligonucleotides. This technology has been previously described, and is based on the electrosynthesis of a conducting polymer film bearing oligonucleotides [8, 9]. The sites are very well defined (50 µm) and the quantity of probes is electrically controlled. In such conditions, a fast read-out system allow quantitative measurement *(figure 3)*.

The MICAM™ chip has a 4 mm^2 active multiplexed device. The 50-µm wide microelectrodes are arranged in a rectangular matrix of eight rows and 16 columns. Each electrode can be selected individually. The silicon chip is integrated into a package that is compatible with both the electrocopolymerization step and the biological analysis. The copolymerization process allows the formation of a homogeneous film (20 nm thick). The amount of hybridizable oligonucleotides grafted onto each electrode has been measured at 200 fmol/mm^2. The functionalization of the gold electrodes by electrocopolymerization allows the formation of a controlled thin-film ODN-pyrrole which is very stable. The homogeneity of this technology is very good and will be easily improved when developed at the industrial level *(figure 4)*.

A second-generation DNA chip is built on a 3-D silicon-structured substrate, using dispensing addressing and electrochemical grafting in 300-picoliter wells



CV # 3%

chip with 128 probes

K-Ras CT, 26 mers, phycoerythrine marker

Figure 4. Homogeneity of the MICAM™ technology.

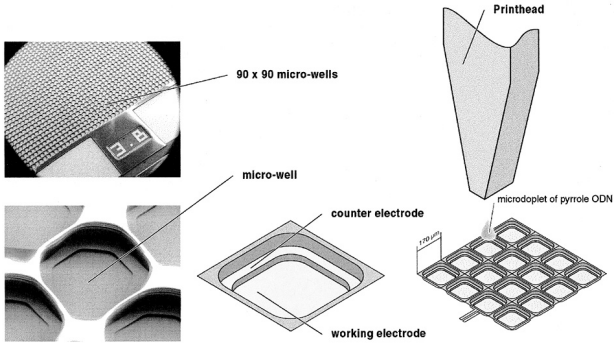


Figure 5. The extended MICAM™ technology.

(figure 5). The advantages of this approach are homogeneous probe density at the electrode surface, highly defined contour of each spot and easy readout of the chip, as each well is in a predefined position and size. DNA chips bearing 8 100 wells are currently being manufactured by CEA (Grenoble, France).

The development of such chips requires fast, adjustable, large dynamic read-out systems. CEA/Leti is also engaged in the development of such elements. *Figure 6* presents an example of new ideas for developing adequate readers. There are no mechanical parts in this system and in such conditions, the readout time is expected to be very fast. Other readers are developed on the basis of a CD reader and are very compact. They integrate miniaturized laser sources and are glass-substrate oriented. The main advantages of this technique are to improve precision, speed of reading and cost of the system.

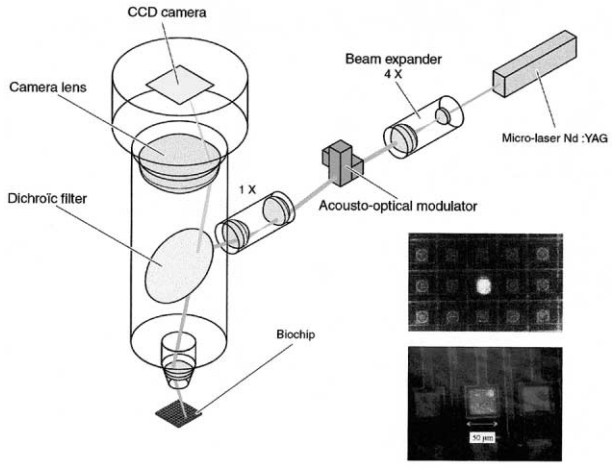


Figure 6. Laser Induced Fluorescence Image.

APPLICATION IN ONCOLOGY

Among other potential applications, we are more especially investigating the clinical diagnosis of gene mutations in cancer and the management of adequate treatment. A rapid and large-scale method to detect K-ras gene mutations in tumor samples has been adapted [11].

The K-ras proto-oncogene is altered by point mutations on codon 12, 13 or 61 in a wide variety of tumors (detection of K-ras mutations enables the understanding of cancer biology and pathogenesis) [12]. These alterations have a clinical relevance, as they provide information for early diagnosis and prognosis. According to the clinical implication of the K-ras gene in human tumorigenesis and its potential role as a target for novel therapeutic approaches, reliable methods are needed for the analysis of the K-ras sequence in clinical samples.

The technical development and the use of this format assay for the detection of K-ras mutations in DNA from human colorectal carcinoma has been described in [10, 11].

Genotyping human colorectal DNA samples

To demonstrate the reliability of the procedure, a blind study was conducted on DNA from 75 different patients with colorectal cancer. The only K-ras codon 13 mutated allele found corresponded to GGC (Gly) and GAC (Asp). The genotypes scored by the polypyrrole DNA chip assay were 100% in agreement with conventional DNA sequencing results. All the K-ras codon 12 mutations detected by direct sequencing were positive by polypyrrole DNA chip analysis, and no false-positive result was obtained with the methodology developed. Moreover, three patients displayed a K-ras codon 13 mutation detected by direct sequencing, and no non-specific hybridization signal was observed on the electrodes corresponding to K-ras codon 12 mutated sequences. *Figure 7* shows an example of the data obtained from direct sequencing and DNA chip analysis for wild-type (GGT) and mutated (GG/AT) DNA. In both samples analyzed, all controls gave the expected results. Particularly, the PC probe gave a strong positive signal, whereas the PP control was negative. Moreover, no background was observed on the gold

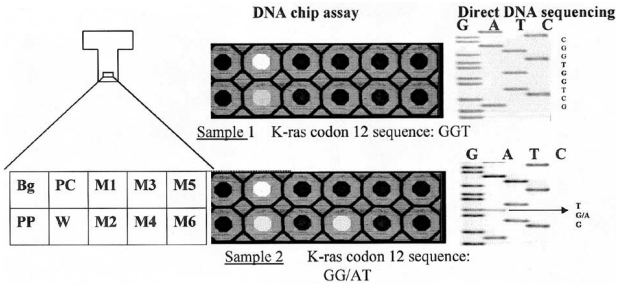


Figure 7. Analysis in oncology with MICAM™ chip on K-Ras codon.

non-polymerized microelectrode Bg. The genotype assignment of K-ras codon 12 by DNA chip analysis clearly distinguished homozygous and heterozygous sequences.

The reliability of hybridization on the ODN-chip depends largely on both the quality of the surface-bound oligomer and the type of ODN–solid support liaison. The ODN were characterized and purified by HPLC before grafting. The functionalization of the gold electrodes by electrocopolymerization allows the formation of a controlled thin-film ODN–pyrrole which is very stable. The stability of the ODN–polypyrrole surface is also compatible with NaOH, and regeneration of the ODN chip after a complete run (hybridization, washing and detection) is possible without significant loss of signal. The 5'-end decathymidine linker makes it possible to increase the hybridization signal by improving ODN accessibility. Moreover, the 3'-end of the grafted ODN is free, and extension by polymerases of the fixed ODN could be envisaged.

The kinetic reaction is as fast as the volume of reactions performed in the micro-chamber is low: 10 µL. Starting from genomic DNA, the detection of point mutations can be accomplished within less than four hours. Research is being carried out on developing an automatic station for the hybridization and analysis steps.

The DNA chip based on electrocopolymerization on gold electrodes gives a support with a low autofluorescence; this allows the detection of less than 10% of mutated sequences within a mixed mutant/wild-type population.

In order to cover the detection of the totality of K-ras mutations, further research is being made on the screening of codon 13 and 61 by using multiplex PCR. Moreover, as 128 electrodes are available we can expect that it will be possible to analyze other genes of interest whose mutations are implicated in the development of cancer. The unambiguous discrimination among the seven potential alleles in the same region suggests that this technology may be useful in multi-allelic gene analysis. Finally, a quantitative application of this silicon device that we have developed is conceivable, since the amount of grafted ODN is perfectly controlled.

CONCLUSION

Quantification of hybridized array images remains a tedious process (due essentially to difficulties in spot detection and inter and intra-spot variability), optimized clustering algorithms have hardly kept pace with the flux of data obtained from the chips, and integration of the results with information stored in biological databases is still a challenge, particularly in the case of mammalian studies.

DNA chip technology has come a long way, but it will continue to develop as new ideas, concepts, and disciplines join this multidisciplinary field dedicated to a better understanding of nature and the living being.

In order to be able to provide quantitative analysis in the field of genomics, we must consider all the steps of such an analysis. Lab-on-a-chip is consequently a very important device which include various functions (mixing, heating, cooling, detection). Its development is complex and will certainly take few years before a large penetration of market is seen, but the actors are as becoming numerous and varied as the potential market is huge.

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