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# APPLICATION OF MICROTECHNOLOGY IN BIOTECHNOLOGY. MICROARRAY ANALYTICAL SYSTEMS – AN OVERVIEW

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The evolution of modern biology, that recently entered the genome era, requires faster and cheaper analytical systems. Techniques as microlithography, micromachining, laser induced fluorescence detection, microfluidics contributed to the development of the DNA microarray technology, having as result complex analytical systems able to perform hybridization of nucleic acids in an array format on small bidimensional surfaces, and also to process huge quantities of information. The most significant achievements worldwide obtained are presented illustrating the state of the art in this domain. In order to automate the fluidic process involved in the DNA hybridization three micromachining techniques, slightly differing from each other have been approached by the authors team. Reservoirs with volumes ranging from 1nl to 2 μl in different materials have been obtained by means of reactive ion etching of polyimide, anisotropic etching of silicon respectively an optimised wet etching of borosilicate glass as well as a low temperature bonding of borosilicate glass on silicon nitride. One obtained device has been tested in a Laser Induced Fluorescence detection set-up.

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## 1. Introduction

A half century after the discovery of the double helix, the completion of sequencing the human genome marks the entrance of modern biology into the genome era. The use of this kind of data corresponding to humans and model organisms will lead to important advances in understanding genetic-based diseases as well as their healing. As a consequence, new research disciplines were born, like pharmacogenomic, pharmacogenetics, molecular diagnosis. These new disciplines are requiring new analytical instruments, able to process the huge information quantity provided by the human genome. Among the branches of analytic research, the most involved one in miniaturization is the analysis of nucleic acids. The polynucleotides are widely used as molecular probes thanks to their selective-pairing capacity by forming of hydrogen bonds, on antiparallel strands. So, Adenine pairs with Thymine, Guanine pairs with Cytosine and therefore are called complementary to each other. This selective pair capacity of the nucleic acids became the basis of "DNA-chip" in genetic diagnosis and genomic analysis that are utilising miniaturised arrays of polynucleotides probes able to reveal, through hybridization, the content of a specific gene domain. Thus, a new technology called "DNA microarray" was born promising to monitor the whole genome on a single chip so that researchers can have a better picture of the interactions among thousands of genes simultaneously.

The miniaturisation of such an analytical system implies not only the huge data processing but also the decreasing of the time and costs, together with the process automation.

## 2. State of the art

In nowadays the above mentioned arrays are obtained by means of direct chemical synthesis of a high number (greater than 100 000) of oligonucleotides probes on appropriate substrates [1-11], using

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techniques already developed by the microelectronics industry. Thanks to a protective photolabile group it is possible to activate square zones of 100-400 $\mu m^2$ using photolithographic masks, allowing the synthesis of an enormous number of oligonucleotides in a low number of steps, like in the system developed by Affymetrix, Inc. [12] and presented in Fig. 1. The number of steps (4N) depends only on the length (N) of the nucleotides to be synthesised, being independent of the total number of oligonucleotides that are to be synthesised. This high analysis power at high costs has as advantage the potential mass production. The obtained devices are analysed by means of laser scanning and fluorescence analysis These 'DNA-chip' may be obtained even without the need of masks, using instead of them micro-mirrors able to create a completely programmable virtual mask, as in the Digital Optical Chemistry System (DOC), developed by the Center for Biomedical Inventions, University of Texas Southwestern Medical Center at Dallas [13] (see Fig. 2). This fully automated system synthesizes DNA directly on a glass surface using light-directed chemistry. Spatially controlled projection of light determines which sequence of bases is created in each region. To spatially control the projection of light, DOC uses Digital Light Processing®, a technology developed by Texas Instruments. The light is projected with an array of microscopic mirrors, each of which is under computer control. The DOC approach affords rapid, flexible, and economical synthesis of custom-designed, high feature-density oligonucleotide arrays.

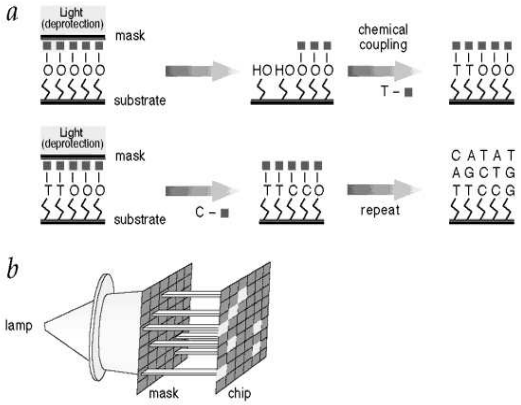



Fig. 1. Affymetrix analysis technique using 'DNA-chip' [12] (with Nature's copyright permission- http://www.nature.com).

In a similar, but most traditional way, Agilent, in collaboration with Rosetta InPharmatics, has developed an analytical system based on inkjet head technology (field in which HP, mother of Agilent is a world leader). This system is based on the micropositioning of the micro-heads that are dispensing the monomers generating the oligonucleotides probes [14].

Other approaches are based on the microdeposition of fragments of oligo- or poly-nucleotides previously synthesised or selected. These methods have a minor operational potential being physically limited by their own parameters of positioning the micro-quantities of material. However in most diagnostic applications the quantity of information gathered in this way is absolutely superior to that obtained by standard methods and it is more than sufficient for the mutation or expression [15-19].

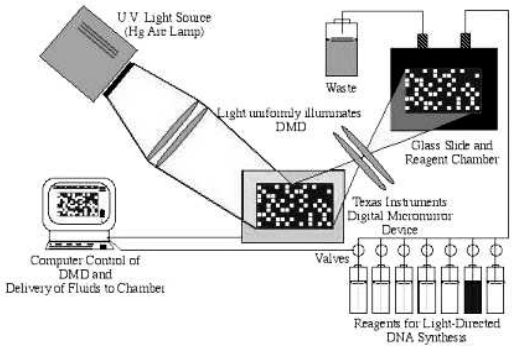



Fig. 2. 'DNA-chip' approach using the Digital Optical Chemistry System, at the Center for Biomedical Inventions, University of Texas Southwestern Medical Center at Dallas [13] (by the courtesy of American Chemical Society).

Potentially more innovative approaches are foreseeing the microfabrication of electrodes arrays able to speed up the hybridisation process (as those developed by Nanogen Inc., CA) [20, 21] (see Fig. 3). The time required by the hybridisation process is considerably shortened by increasing the analyte concentration up to several hundreds of times. This is possible using an electric field in order to move the charged DNA molecules, as the phosphodiesters groups contained in the polynucleotides are negatively charged. Applying an appropriate potential difference between two or more electrodes one obtains the migration of DNA molecules towards the desired electrode. Nanogen is offering today to the market a system able to utilise this principle in order to lead even the clinical molecular diagnosis analysis.

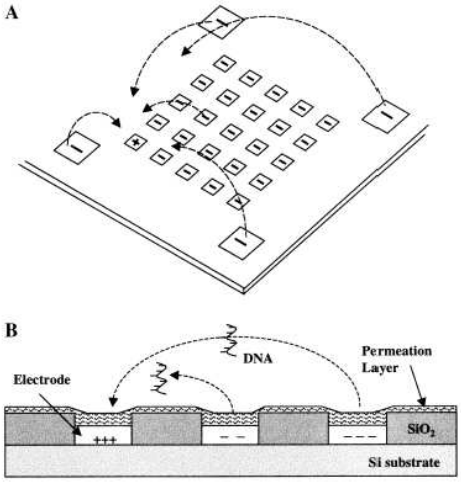



Fig. 3. A) Basic concept for electrophoretic transport of charged molecules (DNA, RNA, proteins, etc.) on an active microelectronic array. The electric field generated by the underlying microelectrodes causes the electrophoretic transport of the molecules to any location on the array surface. B) Cross section of the active microelectronic array. The electrodes are platinum with either platinum or gold wire leads. The permeation layer is a hydrogel (such as agarose or polyacrylamide) that either contains or is impregnated with an affinity or a coupling agent (streptavidin) through which DNA probes (biotinylated oligo-nucleotides (or other materials can be attached [21].

The above mentioned technologies have received a substantial financial support in USA both from the public and from the industrial side. The big pharmaceutics companies have understood from the very beginning the potentiality of these technologies and have led their investments in this field. A significant example is Glaxo that have acquired a strong share participation in Affymetrix in order to obtain a DNA-chip dedicated to rapid analysis of HIV genome, utilisable during the antiviral treatment for tracing the mutations of the genome itself that are leading to resistance. The clinical evidence of resistance is by far subsequent the molecular evidence of resistance, allowing thus to know the exactly moment when the pharmaceutical therapy may be changed.

Not only the pharmaceutical companies have invested in this field but also the big electronic companies such as the already cited Texas Instruments or Motorola (that has invested 250 millions of dollars acquiring the know-how from small start-ups).

In Italy, ST Microelectronics has initiated the same way. The Italian situation is much more delayed on one side due to the absence of a strong industrial presence in the pharmaceutical domain and on the other side due to the low investments in the microtechnology domain. Only recently the public financing has addressed to this field. During the last four years a large multidisciplinary group has grown, around the nucleus formed by LITA in Segrate (Interdisciplinary Laboratory of Advanced Technologies), ITB-CNR (Institute of Biomedical Technologies), University of Milan (Department of Sciences and Biomedical Technologies). Thanks to the financial supporting from the CNR in the frame of the project 'Biotecnologie' other researchers and technologists have joined the group involving the Centre of Quantum Electronics and Electronic Instrumentation (CEQSE-CNR) from

Milano (laser and optics), S. Ana Superior School from Pisa (mechatronics and microfluidics), Universita di Milano Bicocca (informatic analysis), Institute of photonics and Nanotechnology (microfabrication). Systematic studies of the oligonucleotides quality and of the array preparation have been performed, allowing thus the obtaining of usable devices [22-27]. An important achievement is the development of new polymer based chemical platforms, a crucial point on the way to obtain high quality microarray (see Fig. 4).

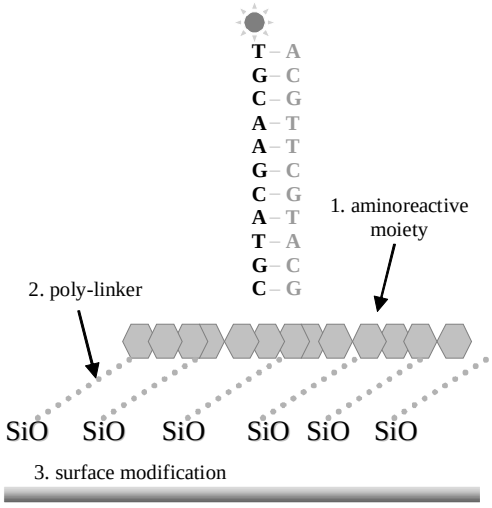



Fig. 4. Hybridisation on a chemical platform by means of a microarray of oligonucleotides probes. Two devices have been obtained: A)1.: carbodiimide / $NH_6$; 2. acrylamide -co-acrilic acid; 3. :APTES, and B) 1. PDC; 2. Poly-L-Lysine; 3: GOPS [31].

A special attention has been paid to the development of a system with arrayed electrodes [29,30,31]. Devices that conceptually are reducible to the Nanogen philosophy have been micromachined. Materials as polyimide, borosilicate glass and respectively silicon have been used for obtaining the reservoirs. Technological flows specific for each material have been approached. In the first step a test mask has been used for structuring the reservoirs.

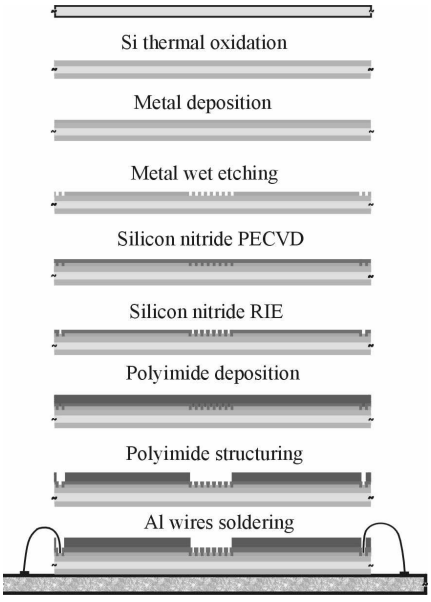



Fig. 5. Main technological steps for manufacturing the electrodes array in a polyimide reservoir [29].

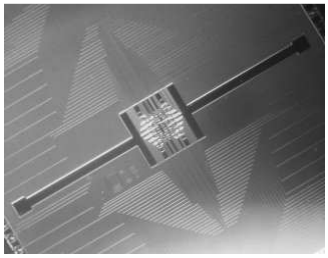


Fig. 6. The polyimide reservoirs; Stereo micrograph x 10 [30].

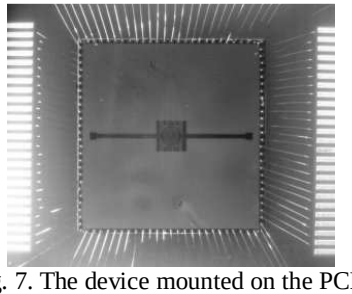


Fig. 7. The device mounted on the PCB; Size 5 × 5 $cm^2$. Stereo micrograph x 7.5 [30].

### 3. Micromachining of the reservoirs with arrayed electrodes

For obtaining the polyimide reservoir the flow presented in Fig. 5 has been followed. Optical lithography was used for patterning the resist. This flow includes: thermal growth of a 4000 Å thick layer $SiO_2$ evaporation of a 3000 Å Au layer over a 200 Å Cr buffer layer, structuring of the 7 × 7 electrodes array, of the 4 reference electrodes and of their corresponding pads and interconnections of 8 μm width, by wet etching of metal layers through an optically patterned resist, plasma enhanced chemical vapour deposition (PECVD) of a 2000 Å silicon nitride layer as insulator of the interconnections, reactive ion etching (RIE) of the silicon nitride layer for opening the contact windows, spin-on deposition of Olin probomide 114 A polyimide, RIE of polyimide layer through an optically patterned resist. The main reservoir allows a volume of 16 nl and has a depth of 5 μm(see Fig. 6). The 12 × 13 $mm^2$ chip mounted on the PCB is presented in the Fig. 7. The electrodes are connected to the external pads by soldering aluminium wires. Using slightly different technological flows, reservoirs deeper than 150 μm and with volumes up to 1 μl have been obtained in glass or in silicon. The second flow (Fig. 10), that uses optical lithography too, includes the microstructuring of borosilicate glass cover and its bonding over the electrodes array (Fig. 8). For glass microstructuring an optimized [32] deep wet etching process has been used through a Cr-Au-resist mask, and a reservoir depth of 170 μm has been achieved. The openings necessary for the connections with the external capillaries have been structured by means of the same deep wet etching process applied on the back side of the glass cover. The bonding of the borosilicate glass cover over silicon nitride chip has been accomplished using a poly-urethane adhesive (Loctite® product 3301). The third flow (Fig. 11), designed for microstructuring the silicon reservoir, uses the electron beam lithography. The electrodes array and their interconnections are thus patterned on an evaporation deposited resist containing octavinylsilsesquioxane (produced by Aldrich Chemical) with a resolution of 10 μm. This limits the electrodes density on the bottom of the reservoirs, so that an array of 5 × 5 electrodes and interconnections having a width of 10 μm has been obtained. The same direct writing with e-beam is to be used for opening the contact windows in the silicon nitride layer. The main steps include: anisotropic etching of Si in KOH 30 % solution at 65° C through a patterned $SiO_2$ layer of 5000 Å, metal deposition by evaporation, wet metal etching through a patterned resist layer, $Si_3N_4$ PECVD. A main reservoir of 2000 × 2000 × 200 $\mu m^3$, inlet and outlet reservoirs of 500 × 500 × 200 $\mu m^3$, and channels of 2000 × 300 × 200 $\mu m^3$ have been thus achieved with the test configuration. A configuration appropriate to the automated fluid flow should include the corner compensation. A SEM micrograph of the main reservoir is presented in the Fig. 9.

Fig. 8. The 200 μm deep reservoir etched in glass, covering the electrodes array.

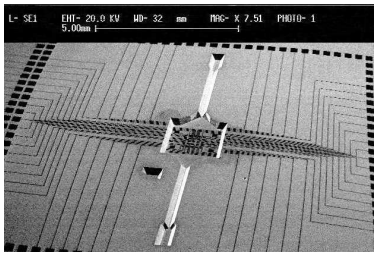



Fig. 9. The 200 μm reservoir etched in silicon[30]; chip size : 12 × 13 $mm^2$ [30].

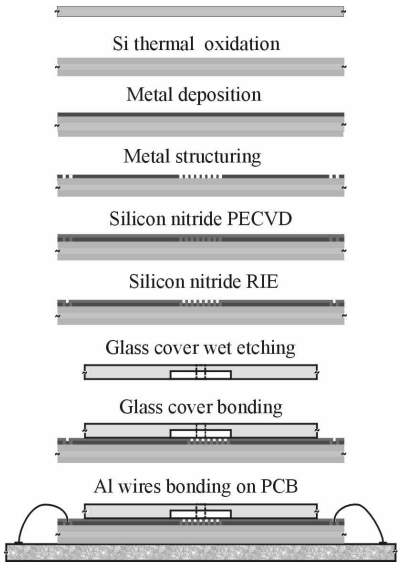



Fig. 10. The main technological flow designed to obtain the reservoir in the glass cover.

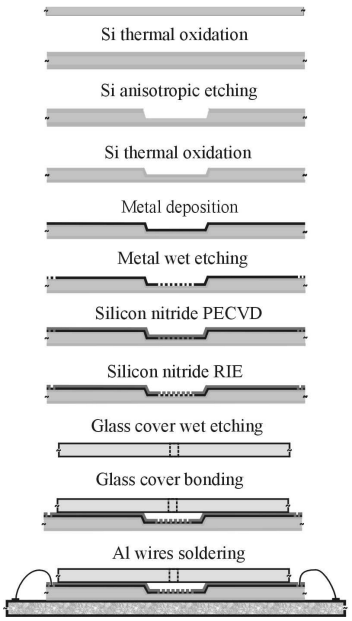



Fig. 11. The main technological steps proposed to obtain a deeper reservoir closed with a glass cover.

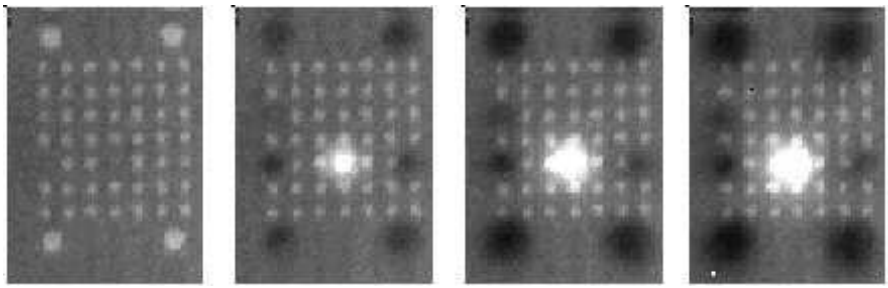
## 4. Functional testing

The problems concerning the detection of fluorescent signals using either intensified CCD cameras or photon counting systems [28] have been investigated. The accumulated experience has allowed the designing of a laser scanning system and later on its manufacturing by Quanta System. The appropriate electronic circuitry and software, for applying the electric potential on the electrodes, have been designed and realised together with the scanning and detection system of laser induced fluorescence (see Fig. 12). The scanner consist of a laser beam focused (20 μm spatial resolution) onto the surface of the chip by means of galvanometric mirrors. We used a Nd-YAG laser (80 mW full power) to excite at 543 nm. Fluorescence was collected at 570 nm using a photomultiplier and proper optical filters.

This whole system has allowed us to experiment the migration technique towards an electrode and the relative identification of the fluorescence signal. We have filled up the measuring chamber with a buffered solution of oligonucleotides marked with cyanine (Cy3, Amersham). The scanning detection showed a weak fluorescence diffused all over the chamber (less than 16 nl). Applying an appropriate potential between the reference electrodes and the working electrode, a rapid electro-migration phenomenon towards the positive electrode has been noticed. In less than 60 seconds most of the oligonucleotides are concentrated over the positive electrode (Fig. 13).

Fig. 12. The laser induced fluorescence detection system built by Quanta System; on the left side one can notice the two galvanometric motors that are driving the deflection mirrors of the laser beam [31].

Our present efforts are concentrated on the preparation of a chemical platform alternative to that one of Nanogen- type, for the functional modification of the electrodes by means of molecular probes that are rendering them specific for certain nucleotide sequence target. Further efforts are dedicated to improve the reservoirs configuration and the input/output capillary connections in order to speed up the fluid flow through the device.



a b c d

Fig. 13. Images of the fluorescent bio-molecules in the reservoir area, captured with a laser scanning system at different time intervals after the electric potential has been applied: a) t=0 s; b) t=15 s; c) t=25 s; d) t=45 s [31].

## 5. Conclusions

A state of the art in the analytical systems based on microarray was presented. Three micromachining techniques are used for obtaining reservoirs in polyimide (RIE), silicon (wet anisotropic etching) and glass (deep wet etching) that are containing the active microarray. An in-house built laser induced fluorescence detection system and the first functional test obtained by the authors were shown. Further efforts are dedicated to develop chemical platforms alternative to 'Nanogen-type' as well as to improve the reservoirs configuration and the input/output capillary connections.

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